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14. ABSTRACT We developed an HPLC method to determine the amount of EGCG encapsulated in the nanoparticles. HPLC analysis showed that chitosan nanoparticles can efficiently entrap EGCG. Also, we developed a method to extract EGCG from the plasma with a recovery value of > 95%. Treatment with EGCG and nanoEGCG resulted in significant inhibition of tumor growth in athymic nude mice implanted with 22Rv1 cells and secreted PSA levels. It was found by transmission electron microscopy that the nanoparticles had a size of around 200 nm. We also found that the release of EGCG from these nanoparticles is very slow in acidic medium. On the other hand, release of intestinal fluid is much faster, and around 50% of EGCG was released in 24 hrs. We next determined the effect of oral nanoEGCG on the growth of tumors in TRAMP mice at 24 weeks of age when the mice had established tumors. Prostate and tumor sizes in nanoEGCG-supplemented TRAMP mice were substantially less than in the control animals. nanoEGCG-supplemented TRAMP mice exhibited a significant reduction in the development of prostate cancer measured 32 weeks on test. There was also induction of apoptosis and inhibition of markers of proliferation and angiogenesis in nanoEGCG-treated groups.					
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INTRODUCTION:

Among all natural agents being tested for their cancer chemopreventive properties green tea has shown promise in preclinical, epidemiological and initial clinical studies. However, absolute clinical success has been hampered due to issues related to bioavailability and toxicity. This study proposes a novel modality involving nanoencapsulation of EGCG for oral consumption designed for prevention and treatment of prostate cancer. Because most biological processes including those that are cancer-related occur at nano-scale, nanotechnology could serve as a potential tool for this unmet need (1). Because of their size range, nanoparticles are very suitable for manipulations at the molecular level, for example cell-receptor binding for site-selective targeting, and localization of encapsulated therapeutics for delivery. With an increasing number of nanoparticle formulations under review by the FDA, and exponentially increasing submission of patents for novel formulations, the outlook for nanoparticle systems in cancer therapy is promising. Nanotechnology will offer significant and increasing improvements in options not only for therapeutic interventions against malignancies but also for disease prevention, a concept that is being addressed through this study (1). In a recent study, we reported significant dose-advantage of polylactic acid-polyethylene glycol (PLA-PEG) encapsulated EGCG (*nanoEGCG*) over non-encapsulated EGCG. NanoEGCG had over ten-fold dose advantage for exerting its pro-apoptotic and anti-angiogenic effects (2).

Oral consumption is the most desired and acceptable form of delivery of chemopreventive agents. Further, one disadvantage of using PLA-PEG nanoparticles is their unstable nature in acidic environment and therefore is not recommended for oral consumption (3). To overcome this obstacle we have been successful in developing an oral formulation of nanoEGCG employing a naturally occurring polymer chitosan which we observed to result in a steady and sustained release of EGCG in the plasma of mice. Our uniquely formulated oral nanoEGCG was synthesized in a mild acidic condition by promoting the interaction of NH₃ group present in chitosan with the phosphate group present in Adenosine 5'-tri-phosphate (ATP). Size distribution and zeta potential of chitosan based nanoparticles encapsulating EGCG was determined by using a Malvern zetasizer (Malvern Instrumentation Co., Westborough, MA). The size of the nanoparticles was found to be in the range of ~200-250 nm in diameter and zeta potential was found to be positive (+ive) as anticipated. The positive charge is an indication of superior muco-adhesive properties of the nanoformulation, which is a prerequisite for any oral formulation. This nano formulated green tea has a significant longer half-life compared to non-encapsulated EGCG. The central hypothesis to be tested in this proposal is that our uniquely formulated nanoEGCG suitable for oral consumption will result in enhanced and sustained availability of EGCG leading to a robust decrease in the effective concentration and prevention of cancer development in mouse models of prostate cancer (PCa).

BODY:

Synthesis of chitosan-based nanoparticles encapsulating EGCG:

Chitosan nanoparticles were synthesized in mild acidic conditions by promoting the interaction of the NH₃ group present in chitosan with the phosphate group present in Adenosine 5'-triphosphate. Briefly, 95 ml of a solution of EGCG (20 mg/ml of DI water) was added to 5 ml of 1% water-soluble chitosan and stirred for 1 hour. Next, 1 ml of a triphosphate disodium salt (20 mg/ml in DI water) was added drop by drop, with constant stirring. The entire solution was then sonicated for about 30 seconds using a probe sonicator, and allowed to stir for another 4 hours

(approx). This solution, containing EGCG nanoparticles, was dialyzed to remove the impurities and the free EGCG using a 100 kD cut-off dialysis membrane. This solution was then lyophilized to get the nanoformulation in powdered form. This was then redispersed in DI water for further use.

Size measurement by Dynamic Light Scattering (DLS):

Size distribution of chitosan-based nanoparticles encapsulating EGCG in aqueous dispersion was determined by using a Malvern zeta sizer (Malvern Instrumentation Co., Westborough, MA, USA). 2 ml of chitosan-based nanoparticles encapsulating EGCG were placed in a 4-sided, clear, plastic cuvette and analyzed directly at 25°C. The size of the nanoparticles was found to be less than 200 nm in diameter (Figure 1).

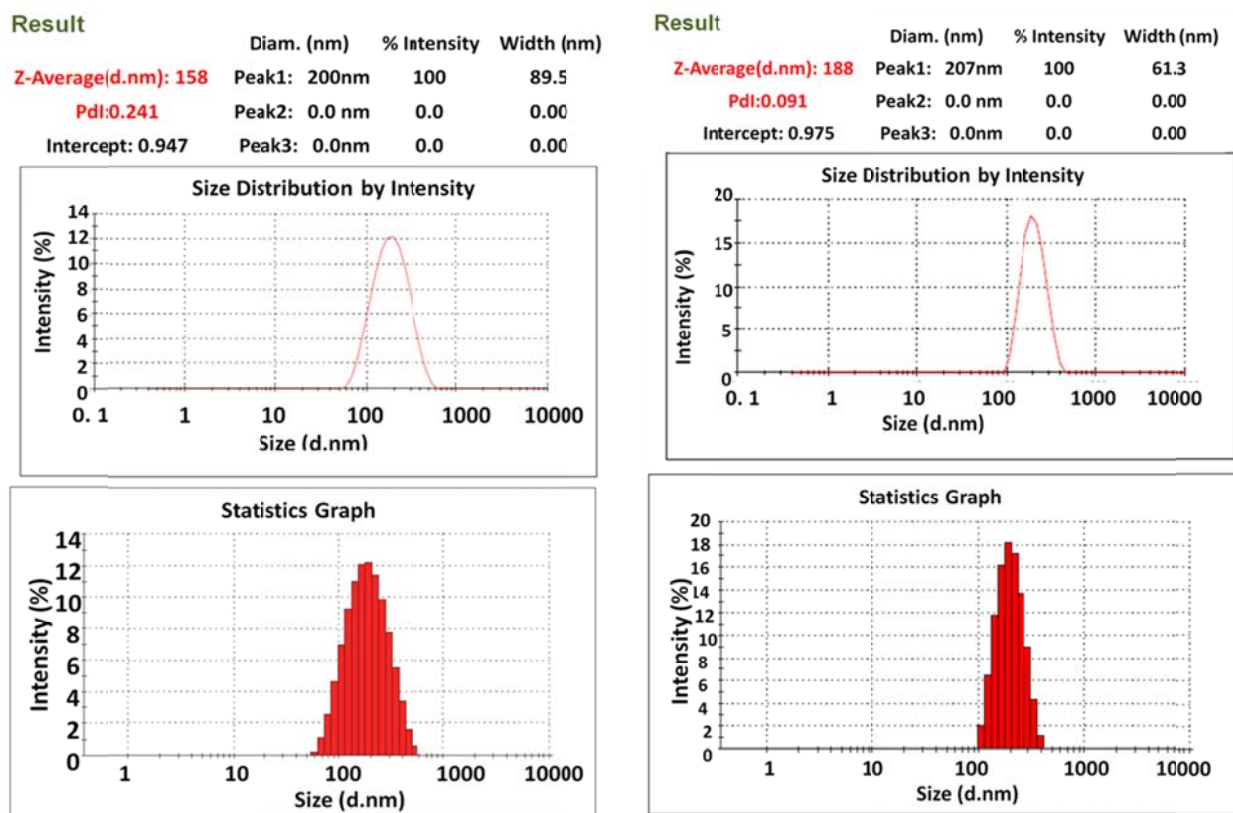


Figure 1: Size measurement by DLS of A) chitosan-based nanoparticles encapsulating EGCG, B) void chitosan nanoparticles.

Analysis of amount of EGCG in the nanoformulation: The amount of EGCG was determined by disintegrating the nanoparticles and measuring the EGCG by HPLC. This also helps in determination of the entrapment efficiency of EGCG in the nanoformulation. First, a calibration curve was obtained with a standard EGCG solution as shown in Figure 2.

The redispersed nanoparticles were disintegrated by adding an acetic acid solution. The entire solution was passed through a filter Millipore centrifugal device of 100 kD cut-off with the help of centrifugation, at around 6500 rpm for 15 minutes to separate the EGCG. The concentration of

the centrifugate containing EGCG was determined using LC/MS. The entrapment efficiency was determined by the following formula:

$$\text{entrapment efficiency} = ([\text{EGCG}]_f) / ([\text{EGCG}]_t) \times 100$$

where $[\text{EGCG}]_f$ is the concentration of EGCG in the centrifugate and $[\text{EGCG}]_t$ is the theoretical concentration of EGCG (meaning total amount of EGCG added initially). Thus, it was found that entrapment efficiency of EGCG was around 75 %.

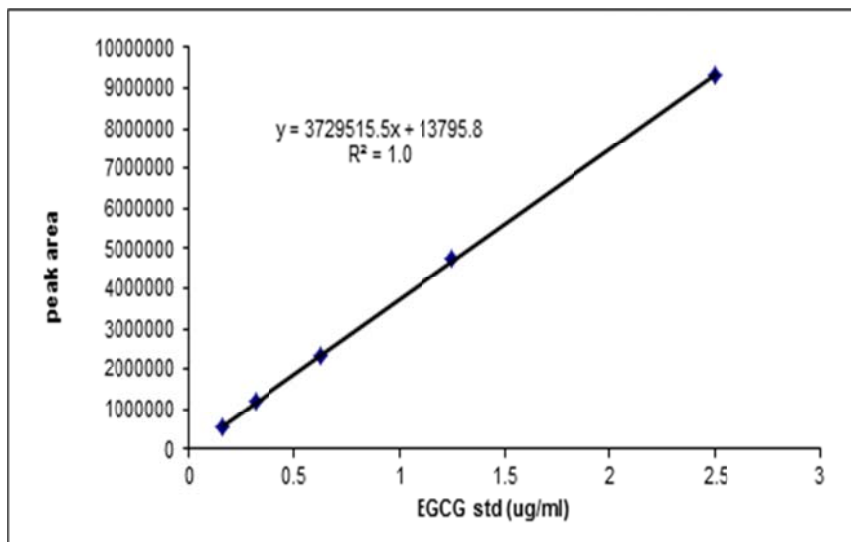
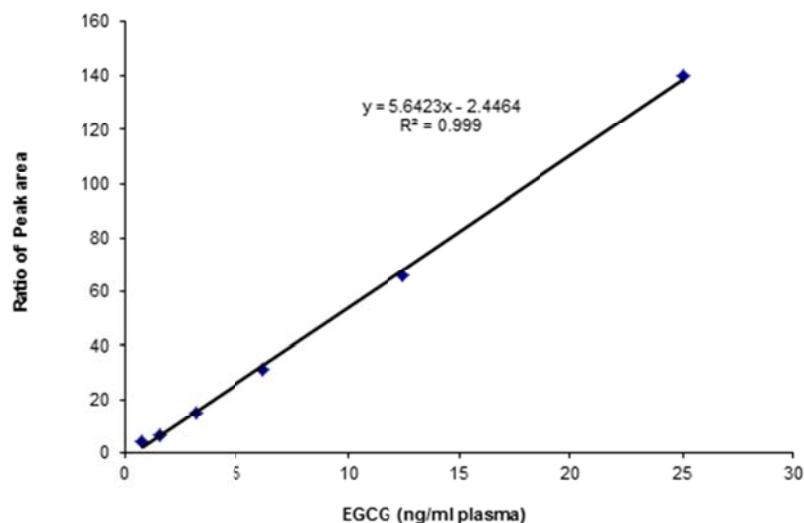


Figure 2: Calibration curve of standard EGCG solution.

LC/MS methods:

Calibration curves: Stock solutions of 0.5 mg/ml and 0.05 mg/ml of EGCG were prepared in 50% methanol (Figure 3). A stock solution of ethyl gallate of 5ug/ml was prepared in the same way in 50% methanol solution. Two calibration curves were obtained to achieve better accuracy. Figure 3A is lower concentration and Figure 3B is the higher concentration of EGCG.

(A)



(B)

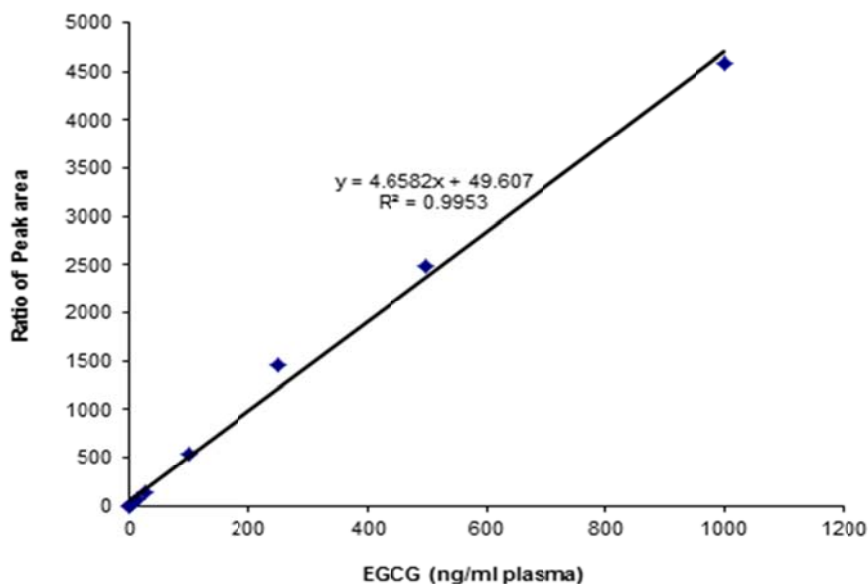


Figure 3: Calibration curve of EGCG spiked into plasma and measured with LC-MS/MS method.

Diluted human plasma (1:3 w/NS) was spiked with EGCG solution to prepare calibration samples ranging from 2 to 1000 ng/ml. 10 μ l of the internal standard was added to each sample before extraction. For each sample, an aliquot of 100 μ l of serum was extracted with ethyl acetate and acetonitrile, and the supernatant was dried. The residual was resuspended with 40% acetonitrile, and 25 μ l was injected for the LC-MS/MS assay. An internal standard was used to normalize the amount of EGCG recovery from the plasma. It was found that the limit of detection (LOD) is 0.05 ng and the limit of quantification (LOQ) is 1 ng/ml in an amount of 80 μ l.

- Shimadzu HPLC includes: LC 20AD pumps; SIL-20AC auto sampler, CTO-20AC column oven and CBM-20A communication bus module;
- Mass spectrometry: Applied Biosystems API 4000 triple quadrupole mass spectrometer, equipped with a Turbo IonSpray source ionizing in the negative mode;
- Isocratic chromatography in MRM mode was performed on a SunFire C18 column, kept at 40 $^{\circ}$ C;
- m/z transitions of EGCG: 457/168.9;
- m/z transitions of ethyl gallate (internal standard): 168.9/124.9

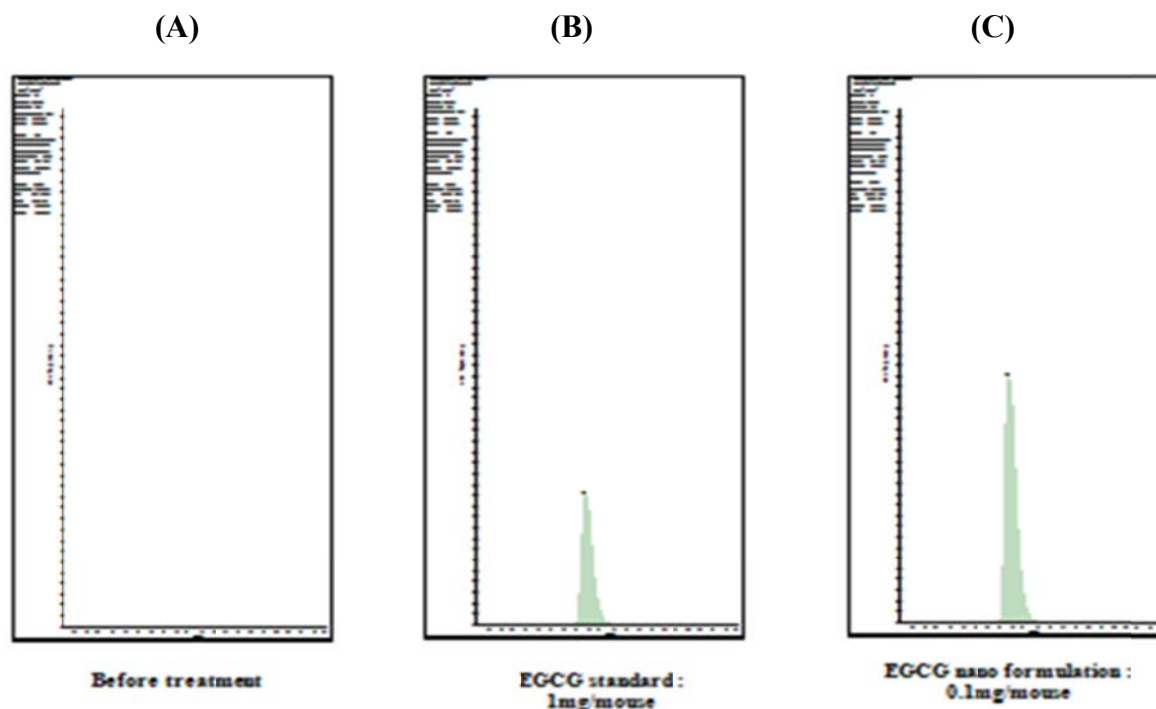


Figure 4: The LC/MS chromatograms of the selected fragment ion of (A) blank serum without EGCG (B) serum EGCG (m/z : 457/168.9; retention time: 1.77 min) from mouse treated with EGCG standard (1 mg) and (C) EGCG nano-formulation (0.1 mg), separately. The mouse blood was collected 2 hours post dosage.

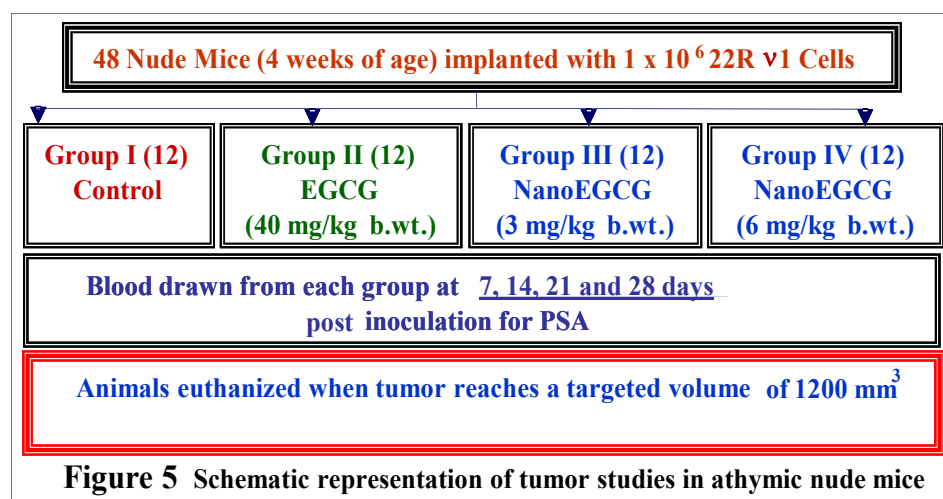
NanoEGCG pharmacokinetics: Oral administration is the most favored route for delivery of chemopreventive agents. However, many agents have low bioavailability because of their poor biopharmaceutical and/or pharmacokinetic profile. As a result, large oral dose is required with conventional delivery systems to attain and maintain the desired levels. However, in majority of such cases high oral dose leads to adverse effects. Various drug delivery systems, each one having its own limitations, have been developed to overcome the hurdles of bioavailability and toxicity. Polymeric nanoparticles offer a great promise for drug delivery and in line with this fact we have successfully developed an oral formulation of nanoEGCG. Therefore, we investigated the pharmacokinetics of oral nanoEGCG in mice to determine the time course of EGCG disappearance from the circulation. **Study design:** Twenty four athymic nude mice were subjected to overnight fasting (39-40), divided into two groups and dosed next morning with either 3 mg/Kg body weight of EGCG or nanoEGCG by oral intubation. Blood was collected either from the retro-orbital plexus or mandible prior to and at 0.5, 1, 2, 4, 8, 16, 24, 48 and 96 hours post intubation. The pharmacokinetics of EGCG disappearance was determined by HPLC analysis of the serum separated from the blood.

Chitosan nanoparticles encapsulating EGCG can be synthesized by gelation methods. The nanoparticle size can be manipulated by different variables like the amount of chitosan, amount of salt. The desired size, which is less than 200 nm, was obtained with the method as mentioned above. The shape and morphology study by transmission electron microscope is in progress. An HPLC method was developed to determine the amount of EGCG encapsulated in the nanoparticles. HPLC analysis showed that chitosan nanoparticles can efficiently entrap EGCG.

Also we have developed a method to extract EGCG from the plasma with a recovery value more than 95%. With the help of an internal standard, the recovered amount of EGCG was measured by LC-MS/MS. The LOD of 0.05 ng and the LOQ of 1 ng/ml indicates the high sensitivity of our method. Figure 4 shows representative LC/MS chromatograms used to calculate the quantity of EGCG in serum by using the calibration curves from Figure 3.

The pharmacokinetics study is under progress. Since this is a time-bound study, blood was collected at selected time-points and the experiment is being repeated and the data analysis is in progress. Other parameters that might need to be optimized in nano-formulation are chitosan, the phosphate salt, and the size of the nanoparticles. These experiments are in progress.

Athymic nude mice tumor study:

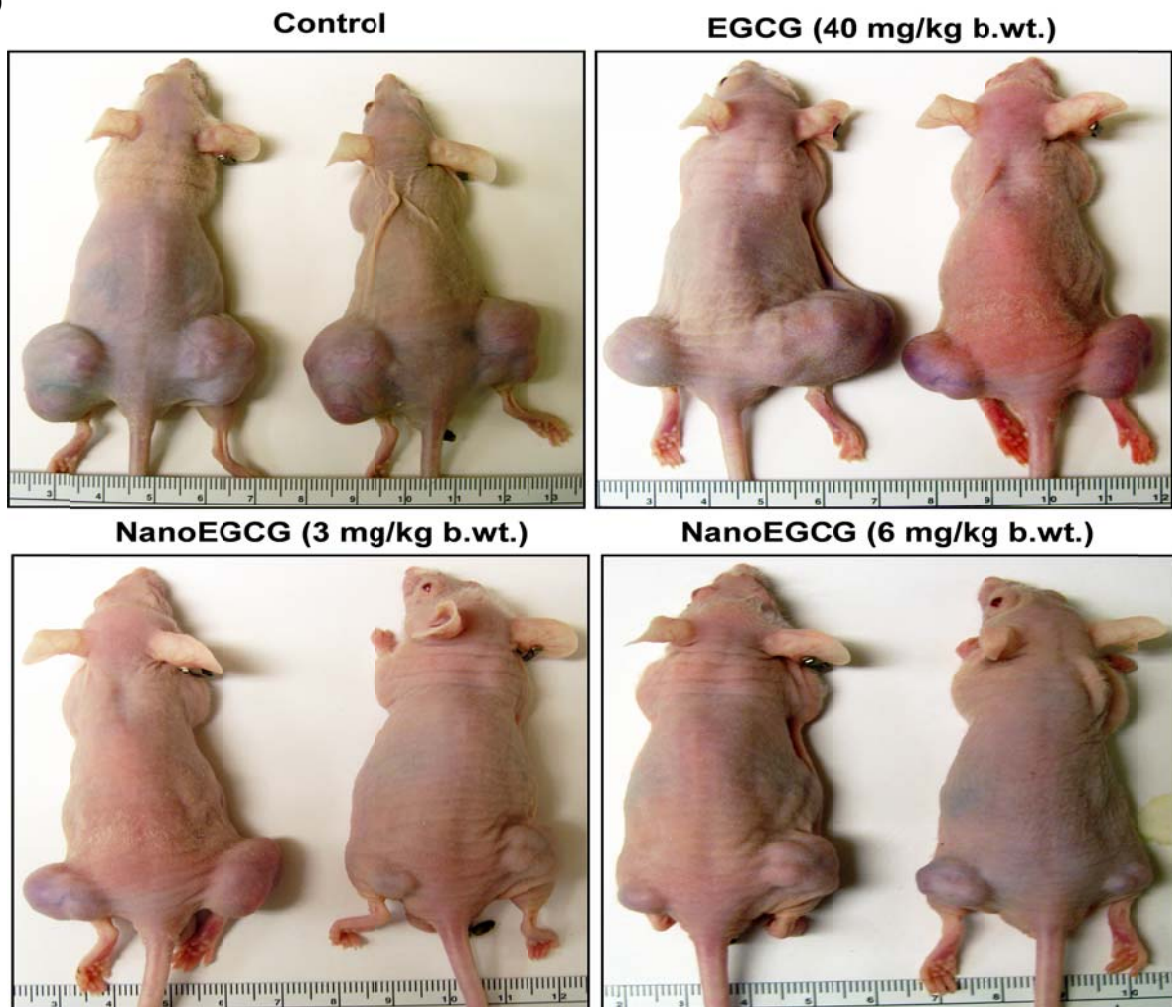


We determined the effect of oral nanoEGCG on the growth of tumors in an *in vivo* situation utilizing athymic nude mice implanted with 22Rv1 cells that form reproducible tumors and also secrete PSA. **Study Design:** Forty eight male athymic nude mice (4 weeks of

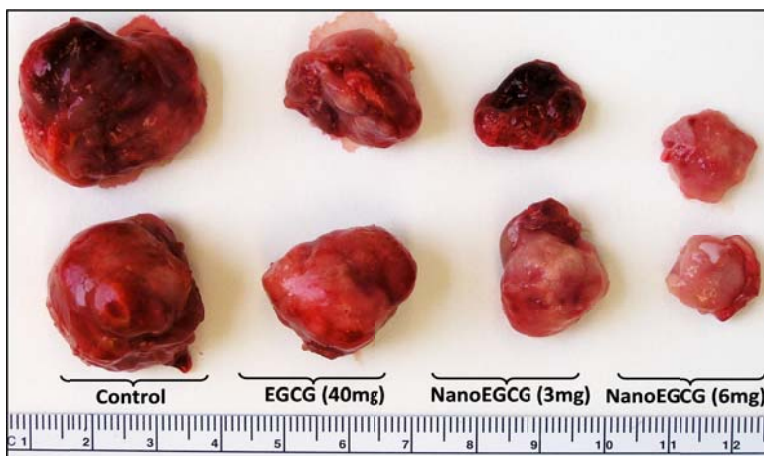
age) were housed four/cage and fed *ad libitum* with autoclaved semi-purified, AIN-76 B-40 diet. A total of 1 million 22Rv1 cells (in 50 μ l DMEM + 50 μ l Matrigel) were implanted by a sub-cutaneous injection on left and right sides, below the shoulders (2 tumors per mouse). **Treatment of nude mice:** Mice implanted with cells were randomly distributed into four groups of twelve each (**Figure 5**). Group I received void nano particles and served as the control. Group II received EGCG (40 mg/kg b.wt). Group III received nanoEGCG (3 mg/kg b. wt) and Group IV received nanoEGCG (6 mg/kg b. wt). Treatments were by oral intubation and started one day post cell inoculation, 5 times a week (Monday-Friday) until tumors reached a targeted volume of 1200 mm³. **Tumor volume estimation:** The tumor size was measured by determining two perpendicular dimensions with calipers, and the volume was calculated using the formula $(a \times b^2)/2$, where a is the longer and b is the smaller dimension. The animals were also be evaluated for body weight, consumption of food and apparent signs of toxicity. At weekly intervals phlebotomy was performed to obtain sera for PSA estimation by ELISA.

NanoEGCG inhibits the growth of human prostate carcinoma CWR22Rv1 cells in athymic nude mice:

(A)



(B)



The treatment of athymic nude mice with nanoEGCG resulted in inhibition of AR-positive CWR22Rv1 tumor xenograft growth. The appearance of small solid tumors was observed in animals of control group 11 days after cell inoculation. This latency period was prolonged to 18 days in animals receiving EGCG (40 mg/kg body wt.) and 25 days in animals receiving nanoEGCG

Figure 6

(3 and 6 mg/kg body wt.). There was significant reduction in growth of prostate tumors in EGCG and nanoEGCG-treated animals as compared to control group (Figures 6-8). As depicted in Fig. 7, tumor growth, as inferred by computed tumor volume, was significantly inhibited in mice receiving EGCG and nanoEGCG. In control group, the average tumor volume of 1,200 mm³ was reached in 32 days after tumor cell inoculation. At this time point, the average tumor volume was 514, 310 and 216 in EGCG, nanoEGCG (3mg/kg body wt.) and nanoEGCG (6mg/kg body wt.) treated groups, respectively. The average tumor volume of 1,200 mm³ was achieved 46, 53 and 60 days after tumor cell inoculation in EGCG, nanoEGCG (3mg/kg body wt.) and nanoEGCG (6mg/kg body wt.) treated groups, respectively (Figures 7 and 8). There was dose-dependent inhibition of tumor growth by nanoEGCG and it was found to be more effective than EGCG.

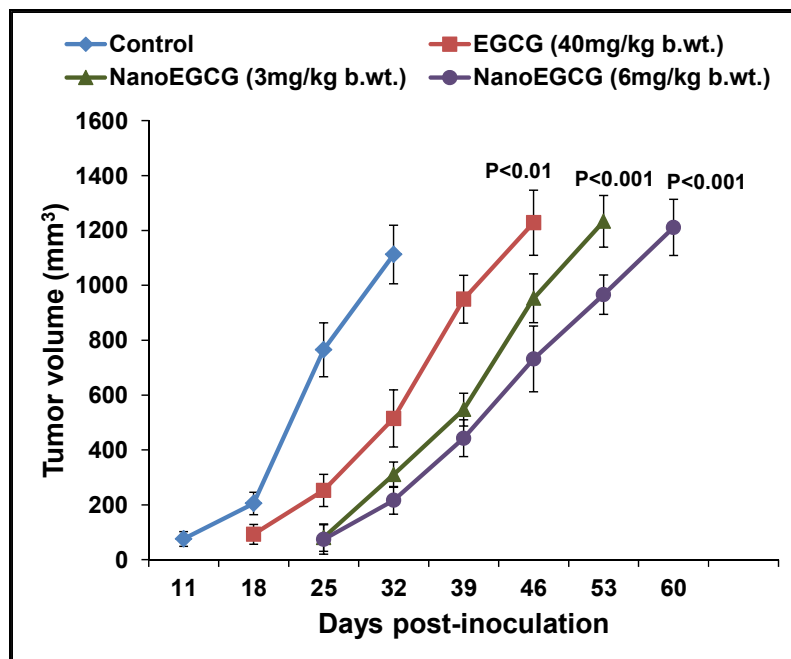


Figure 7

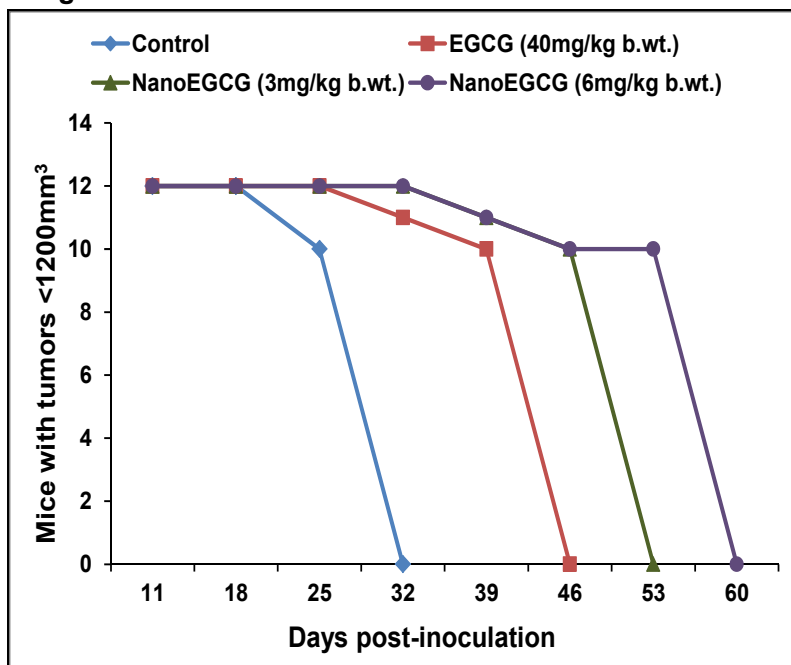


Figure 8

NanoEGCG inhibits PSA secretion in athymic nude mice:

During the course of tumor growth in animals at day 2, 8, 14, 20 and 26 after inoculation, blood was collected through the mandibular bleed. Quantitative sandwich ELISA was used to

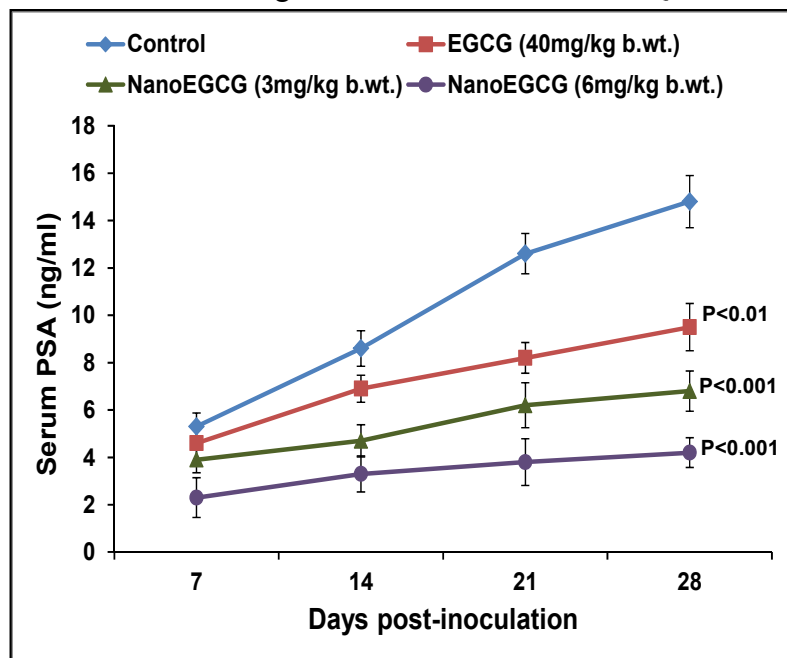


Figure 9

benign prostatic hypertrophy, and prostate cancer (4). In our study, we found that there was significant inhibition of secreted PSA levels by 13-36%, 26-54% and 57-72% in EGCG, nanoEGCG (3mg/kg body wt.) and nanoEGCG (6mg/kg body wt.) treated groups, respectively as compared to the control group normalized to tumor volume (Figure 9). Hence, our results show that treatment of mice with nanoEGCG caused dose-dependent significant decrease in the serum PSA in athymic nude mice and the effect was more pronounced with nanoEGCG than EGCG.

KEY RESEARCH ACCOMPLISHMENTS:

Prostate cancer remains the most common cancer in men in the United States and next only to lung cancer, is the second leading cause of cancer-related deaths in American males.

- We have been successful in developing an oral formulation of nanoEGCG using a naturally occurring polymer chitosan which we observed to result in a steady and sustained release of EGCG in the plasma of mice. This nano formulated green tea has a significant longer half-life compared to non-encapsulated EGCG.
- Through these studies in athymic nude mice, we established the anti-tumor efficacy of oral nanoEGCG vs. EGCG alone. Oral formulated nanoEGCG compared to EGCG resulted in a significant inhibition of tumors in nude mice based on increased bioavailability.
- We also observed a significant dose-dependent decrease in serum-PSA levels in athymic nude mice in nanoEGCG-treated groups.
- Far superior tumor growth inhibitory effects were observed with oral nanoEGCG at significantly lower doses.

- Our experimental design established a dose dependent efficacy of oral nanoEGCG which will guide us on the dose required for further studies in Nkx3.1/Pten and TRAMP mice.
- In future studies, we will investigate if supplementation of oral nanoEGCG will inhibit the development of PIN lesions in the Nkx3.1/Pten mutant mice via modulations in i) pro-inflammatory milieu, and ii) oxidative stress in the prostate. Further, we will determine the preventive and therapeutic efficacy of oral nanoEGCG and to identify the stage of prostate cancer development that is most vulnerable to the anti-cancer effects of oral nanoEGCG in the transgenic TRAMP mice.

CONCLUSION:

The issues related to bioavailability and perceived toxicity of green tea pose a great challenge which are addressed in this study through proposing the use of the novel and uniquely formulated oral nanoEGCG. Nanotechnology will offer significant and increasing improvements in options not only for therapeutic interventions against malignancies but also for disease prevention, a concept that is being addressed through this proposal. It is expected that nanotechnology will continue to have a profound and positive impact on human health. Many nanotechnology-based diagnostic and treatment modalities already are in use, with many others at various stages of pre-clinical and clinical testing. Several nanotechnology platforms hold great promise for diagnosis and treatment of cancer. We propose that nanotechnology will serve as a cornerstone in cancer chemoprevention. Through this study, we propose a novel preventive and therapeutic modality using EGCG that will address issues related to bioavailability, toxicity and dose requirement that are major reasons for its limited success in humans.

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INTRODUCTION:

Prevention of cancer by natural agents is often restricted by the lack of delivery of desired levels in target tissue thus limiting their bioavailability and clinical outcome. Hence, newer approaches are required to improve bioavailability and to decrease toxicity of natural agents to accomplish maximum response. Among all natural agents, green tea has shown promise in preclinical, epidemiological and initial clinical studies, especially for prostate cancer (PCa). Much of the effects of green tea are considered to be exerted by its major polyphenol (-) epigallocatechin-3-gallate (EGCG). We reported significant dose-advantage of polylactic acid-polyethylene glycol (PLA-PEG) encapsulated EGCG (nanoEGCG) over non-encapsulated EGCG (1), for PCa in cell-culture and xenograft model. However, PLA-PEG is unstable in acidic environment and is therefore not recommended for oral consumption, a desired route for chemopreventive agents. We developed a formulation of chitosan-based nanoparticles encapsulating EGCG (CHI-EGCG-NPs). Despite extensive efforts that have been put to prevent cancer through the use of bioactive food components and other agents, lack of delivery of desired levels thus limiting their bioavailability, remains a challenging issue for effective clinical outcome (2-4). There is a strong unfulfilled need in the biomedical community to find novel tools for making prevention and treatment of cancer a success. Therefore, in order to achieve maximum response of a natural agent, novel strategies are urgently needed to enhance the bioavailability, effectiveness and to reduce perceived toxicity. It is expected that nanotechnology will continue to have a profound and positive impact on human health. Many nanotechnology-based diagnostic and treatment modalities already are in use, with many others at various stages of pre-clinical and clinical testing. Several nanotechnology platforms hold great promise for diagnosis and treatment of cancer. We propose that nanotechnology will serve as a cornerstone in cancer chemoprevention. The concept of nano-chemoprevention (i.e. encapsulation of chemopreventive agents in nanoparticles) possesses strong merit and rationale for conducting additional detailed *in vivo* studies in appropriate animal models with relevance to human disease. As with many other nanoparticles, the advantage of using PLA-PEG nanoparticles lies in their high surface area to volume ratio, which presumably allows them to upload more EGCG while maintaining the small size. This is particularly important because the enhanced conjugation of nanoparticles with agents often results in the generation of nanoparticles that are sparingly taken up by the diseased cells, owing to their bigger size and, thereby, undermining their medical usefulness. Also, the therapeutic/clinical importance of PLA-PEG nanoparticles relies on the fact that, being biodegradable, they rarely exhibit any toxicity.

BODY:

Size measurement by Transmission Electron Microscopy (TEM):

The size and morphology of CHI-EGCG-NPs were further examined by transmission electron microscopy (TEM) using a JEOL JEM-100CX transmission electron microscope (JEOL, USA, Inc., Peabody, MA, USA). One drop of the CHI-EGCG-NPs solution was mounted on a thin film of amorphous carbon deposited on a copper grid (300 mesh). The solution was air dried and the sample was examined directly.

As reported last year, CHI-EGCG-NPs were further characterized by TEM. This technique allowed us to directly visualize the CHI-EGCG-NPs. As shown in Figure 1, the nanoparticles have a size of around 200 nm. Additionally, from the TEM picture it is clear that the nanoparticles are spherical in shape.

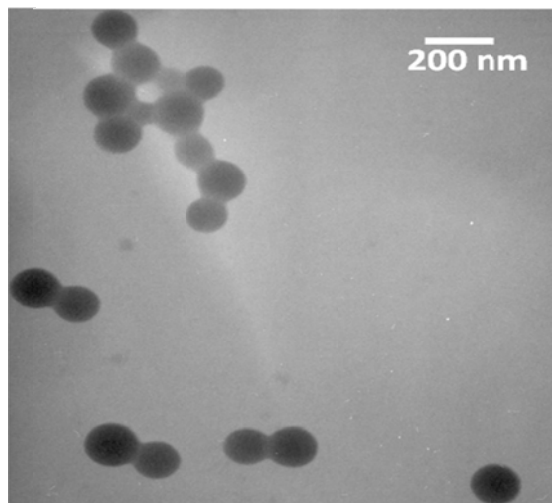


Figure 1: Transmission Electron Microscopy Images showing the spherical size of CHI-EGCG-NPs.

Release kinetics studies:

We have studied the release kinetics of EGCG from CHI-EGCG-NPs in simulated gastric juice and simulated intestinal fluid. For this cumulative release kinetic study, a known amount of nanoparticles encapsulating EGCG was suspended in 15 ml of simulated gastric juice/simulated intestinal juice. The solution was kept in an incubator at room temperature (25°C). At predetermined intervals of time, 500 μ l of the solution was filtered through Millipore centricon tubes containing a 100 KD membrane to separate the released EGCG from the nanoparticles. The amount of free EGCG present in the filtrate was determined by LC/MS. A graphical representation comparing the release profiles in simulated gastric juice and simulated intestinal fluid is shown in Figure 2.

In the case of oral delivery of an active biomaterial/natural product the degradation starts in the stomach, due to the acidic pH. One of the most important factors to be kept in mind for carrier-mediated drug delivery through an oral route is that the carrier system must not degrade/release at acidic pH, releasing the entire drug. At the same time, it has to retain its capability to release the drug at neutral pH. To test the stability and release kinetics of CHI-EGCG-NPs we studied the release kinetics in simulated gastric juice and simulated intestinal fluid. It is clear from the Figure 2 that the release of EGCG from these nanoparticles is very slow in acidic medium; even at 24 hrs only ~10% of EGCG was released. On the other hand, release of intestinal fluid is much faster, and around 50% of EGCG was released in 24 hrs. Then there was a steady release of EGCG. This is strong evidence that these nanoparticles are capable of avoiding degradation in the stomach, they do not release EGCG and they are capable of releasing EGCG in a neutral medium.

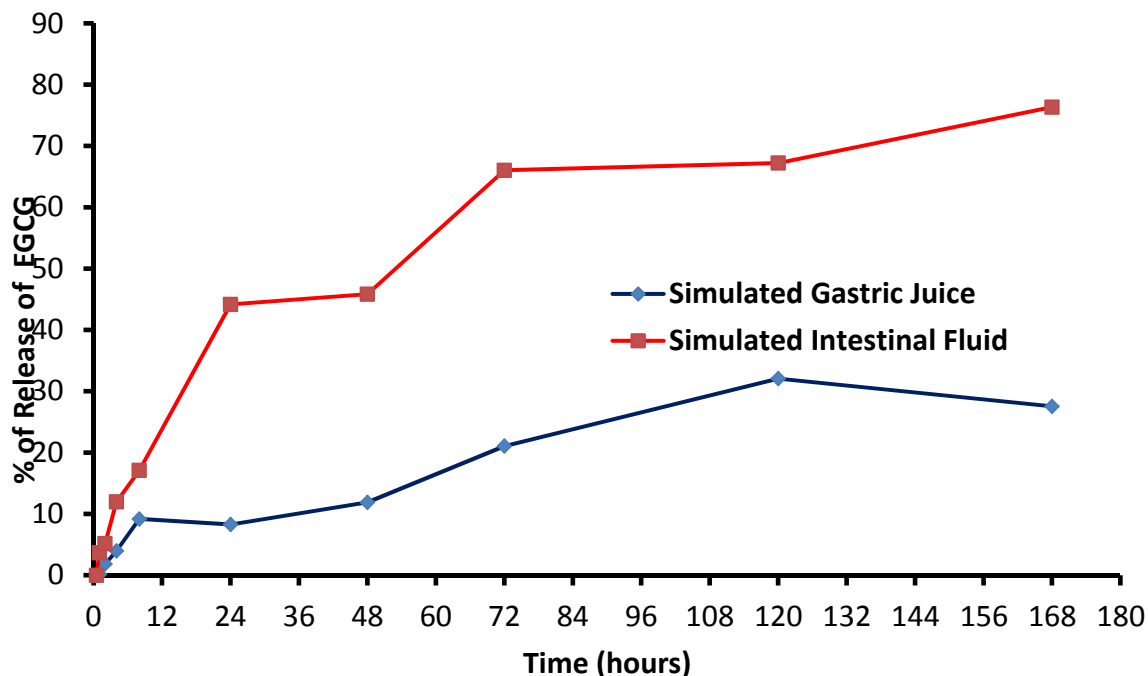


Figure 2: Release kinetics study of EGCG from CHI-EGCG-NPs in simulated gastric juice and simulated intestinal fluid.

Analysis of EGCG by LC/MS

Stock solutions of EGCG were obtained by dissolving EGCG in 50% methanol. The calibration curve for EGCG was obtained by spiking stock EGCG solutions of different concentrations in bovine plasma. At least two calibration curves were acquired to achieve a more reliable technique (Figure 3). With the help of this standard curve, we can measure the amount of EGCG in the range of 0.8-31.28 ng/ml. In brief, the calibration curve was obtained by diluting bovine plasma (1:3 w/NS) and spiked with EGCG solution ranging from 0.8 to 31.25 ng/ml. For sample analysis, 10 μ l of an internal standard (ethyl gallate) was added to each sample before extraction. A total amount of 100 μ l of spiked serum sample was extracted from each sample with ethyl acetate and acetonitrile. The supernatant was dried and the residual was resuspended with 40% acetonitrile, and 25 μ l was injected for the LC/MS/MS assay. In all cases, an internal standard was used to normalize the amount of EGCG recovery from the plasma.

Figure 3 shows the calibration curves obtained from EGCG standard solution at different concentrations (0.8-31.25 ng/ml). These standard curves were used to quantify the amount of EGCG in the mice serum at various time points (of the pharmacokinetics studies). The experiments for the standard curves were in duplicate to test the accuracy of our method. From Figure 3, it is observed that the two curves are nearly identical to each other, indicating our method is accurate.

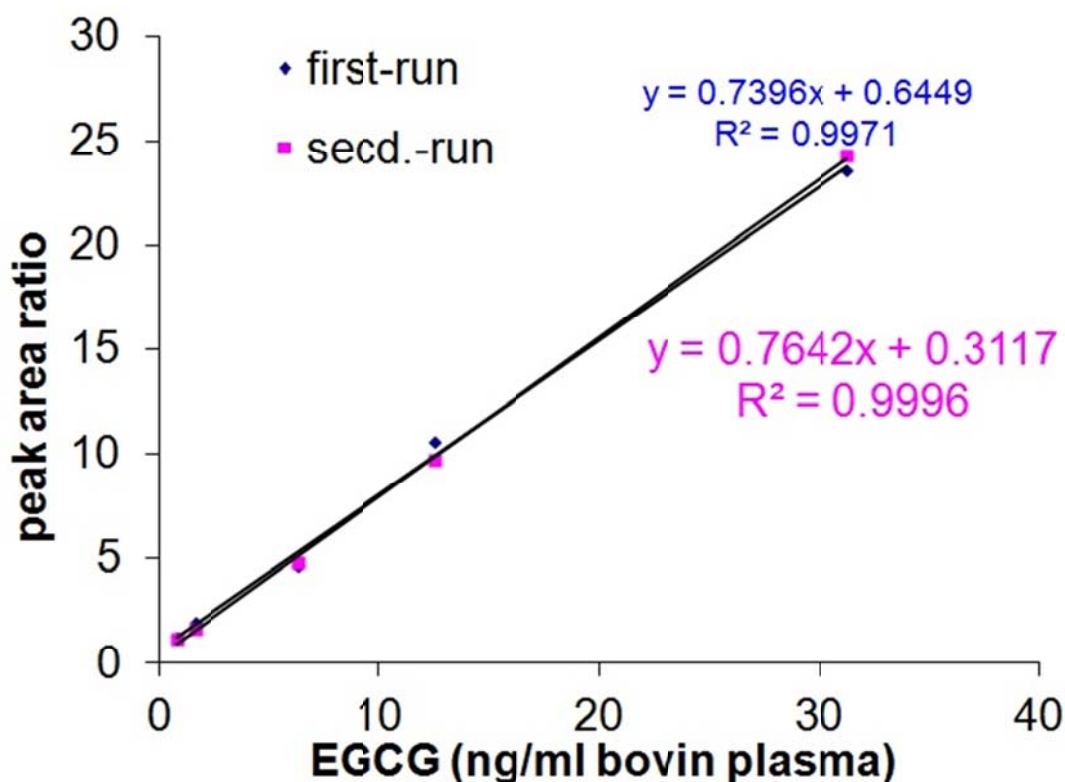


Figure 3: Calibration curves generated with bovine plasma. EGCG ranges from 0.8-31.25 ng/ml. The calibration samples were measured twice.

The HPLC was a Shimadzu Prominence LC system containing a CBM-20A system controller, two LC-20AD pumps, a SIL-20AC autosampler, and a CTO-20AC oven. For the analysis of EGCG, a Waters Sunfire™ C18, 3.5 μ m particle size, 3.0 \times 150 mm column at and an isocratic elution method with mobile phases of 40% acetonitrile and 0.1% formic acid was used. The flow rate was set at 0.35 ml/min and the eluent was directed to the mass spectrometer ion source without splitting. An API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, MDS SCIEX Ontario, Canada) equipped with a Turbo V Source and Turbo Ion Spray was coupled to the HPLC. The mass spectrometer was operated in negative mode and selective ion monitoring was used for qualitative analyses of EGCG (EGCG, m/z 457), and multiple reaction monitoring was used for quantitative analyses of EGCG with mass transitions 457 \rightarrow 168.9 m/z (Figure 4).

Figure 4 shows a typical chromatogram (LC/MS) of EGCG (blue line) in various physiological conditions. Figure 4 (i) shows the chromatogram of standard EGCG solution (12.5 ng/ml); Figure 4 (ii) shows the chromatogram of EGCG in bovine plasma (6.25 ng/ml), and Figure 4 (iii) shows the chromatogram of EGCG in mouse serum (1.17 ng/ml). In all cases, an internal standard of ethyl gallate (red line in the chromatogram) was used for normalization of the data.

All these chromatograms demonstrate our ability to detect EGCG in different physiological conditions at varying concentrations.

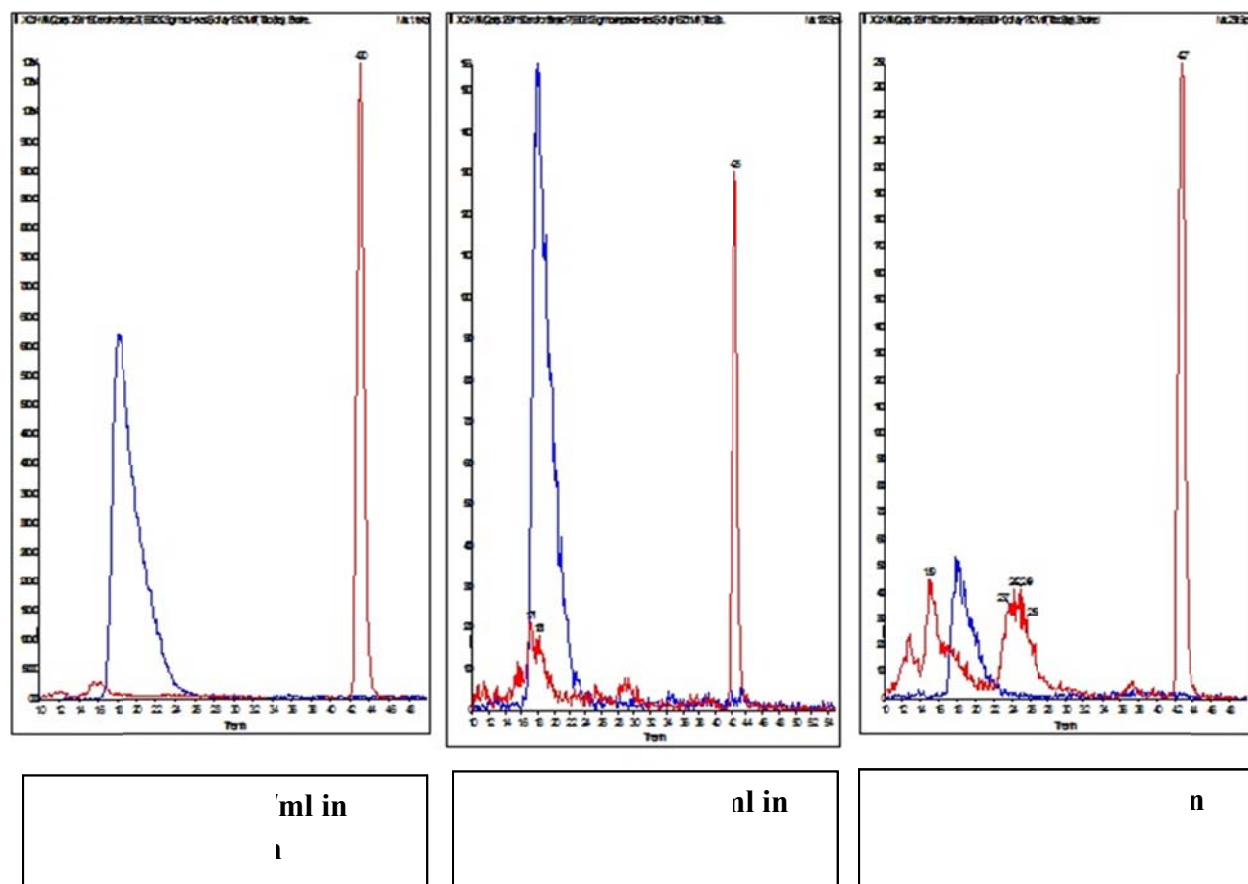
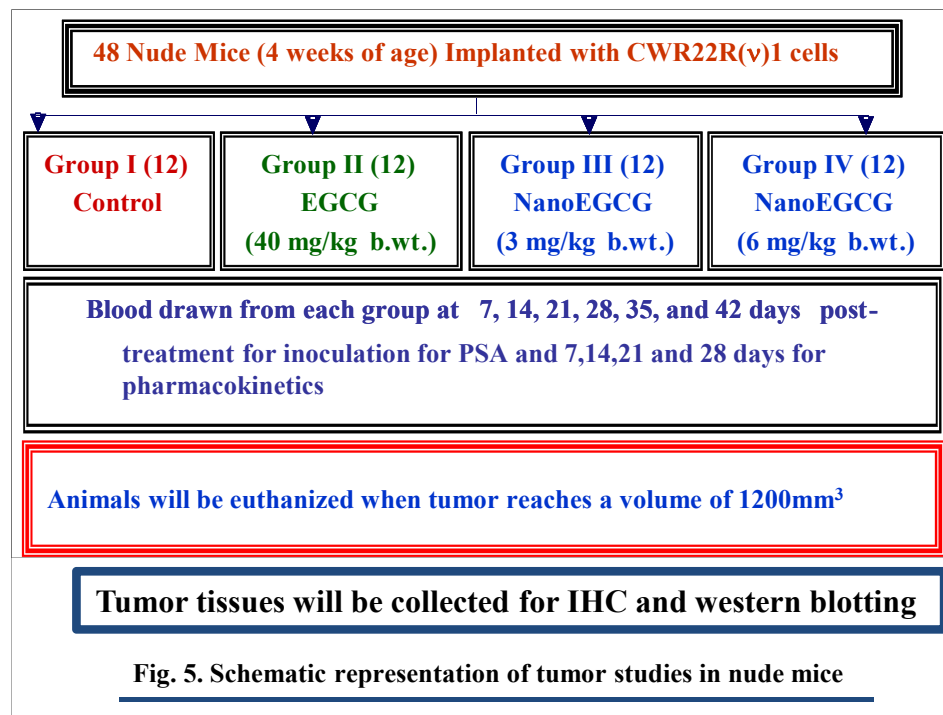


Figure 4: Typical chromatograms of EGCG in various matrixes detected by LC/MS

Athymic nude mice study:



Design: Forty eight male athymic nude mice (4 weeks of age) were housed four/cage and fed *ad libitum* with autoclaved semi-purified, AIN-76 B-40 diet. A total of 1 million 22Rv1 cells (in 50 μ l DMEM + 50 μ l Matrigel) were implanted by a

sub-cutaneous injection on left and right sides, below the shoulders (2 tumors per mouse). **Treatment of nude mice:** Mice implanted with cells were randomly distributed into four groups of twelve each (Figure 5). Group I received void nano particles and served as the control. Group II received EGCG (40 mg/kg b.wt). Group III received nanoEGCG (3 mg/kg b. wt.) and Group IV received nanoEGCG (6 mg/kg b. wt.). Treatments were by oral intubation and started one day post cell inoculation, 5 times a week (Monday-Friday) until tumors reach a targeted volume of 1200 mm³. The animals were also evaluated for body weight, consumption of food and apparent signs of toxicity. At weekly intervals phlebotomy was performed to obtain sera for PSA estimation by ELISA and for pharmacokinetics study. Tumor tissues were collected at the end of the protocol for immunohistochemical and western blotting for evaluation of cell proliferation markers (Ki-67 and PCNA) and apoptotic markers (PARP, Bax, Bcl2 and caspases). **Analysis of serum EGCG concentrations:** Serum samples from EGCG and nanoEGCG treated mice were subjected to HPLC and GC mass spectrometry for analysis of pharmacokinetic distribution of EGCG and bioavailability.

Though we have developed a sensitive method to measure the amount of EGCG levels in blood plasma, there was little detectable EGCG found in the plasma after 7 days (Figure 6). The probable reason may be that the serum was collected too late after the oral administration of EGCG/CHI-EGCG-NPs, and that most of the EGCG was secreted out of the body or was converted to other metabolites.

	dosage (mg/Kg)	code of mouse	EGCG con. (ng/ml)		dosage (mg/Kg)	code of mouse	EGCG con. (ng/ml)
Day-7	control	mouse-1	BLOQ	Day-21	control	EGCG-III-1	BLOQ
		mouse-2	0.14			EGCG-III-2	BLOQ
		mouse-3	0.03			EGCG-III-3	BLOQ
	3	mouse-4	BLOQ		3	EGCG-III-4	BLOQ
		mouse-5	BLOQ			EGCG-III-5	60.18
		mouse-6	BLOQ			EGCG-III-6	BLOQ
	6	mouse-7	BLOQ		6	EGCG-III-7	BLOQ
		mouse-8	BLOQ			EGCG-III-8	BLOQ
		mouse-9	BLOQ			EGCG-III-9	BLOQ
	40	mouse-10	BLOQ		40	EGCG-III-10	BLOQ
		mouse-11	BLOQ			EGCG-III-11	BLOQ
		mouse-12	BLOQ			EGCG-III-12	BLOQ
Day-14	control	EGCG-II-1	BLOQ	Day-28	control	EGCG-IV-1	BLOQ
		EGCG-II-2	BLOQ			EGCG-IV-2	BLOQ
		EGCG-II-3	BLOQ			EGCG-IV-3	BLOQ
	3	EGCG-II-4	BLOQ		3	EGCG-IV-4	BLOQ
		EGCG-II-5	0.91			EGCG-IV-5	BLOQ
		EGCG-II-6	0.55			EGCG-IV-6	BLOQ
	6	EGCG-II-7	BLOQ		6	EGCG-IV-7	BLOQ
		EGCG-II-8	0.12			EGCG-IV-8	BLOQ
		EGCG-II-9	BLOQ			EGCG-IV-9	BLOQ
	40	EGCG-II-10	1.17		40	EGCG-IV-10	BLOQ
		EGCG-II-11	0.16			EGCG-IV-11	BLOQ
		EGCG-II-12	0.14			EGCG-IV-12	BLOQ

Figure 6: EGCG concentration in blood plasma after oral administration of EGCG and EGCG encapsulated nanoparticles (CHI-EGCG-NPs). (BLOQ: below limit of quantification)

Effect of CHI-EGCG-NPs on apoptosis in tumor tissues of athymic nude mice

Protein extraction and western blotting

For western blotting, 30-50 µg protein was resolved over 8-12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% nonfat dry milk/1% Tween 20; in 20 mM TBS, pH 7.6) for 1 hr at room temperature, incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for one and half hr to overnight at 4°C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate obtained from Amersham Life Science Inc. (Arlington Height, IL, USA) and detected by chemiluminescence and autoradiography using Bio-Rad Gel-Doc (Bio-Rad Laboratories Inc., Hercules, CA).

As shown by western blots in Figure 7, PARP cleavage analysis showed that the full size PARP (116 KD) protein was cleaved to yield an 85 KD fragment on treatment with CHI-EGCG-NPs. There was also a significant increase in the protein expression of Bax whereas; the protein expression of Bcl-2 was significantly decreased by CHI-EGCG-NPs in a dose-dependent fashion. We also found the activation of casapase-3, -8 and -9 in tumor tissues of mice treated with both doses of CHI-EGCG-NPs as compared to group treated with EGCG and control (Figure 7).

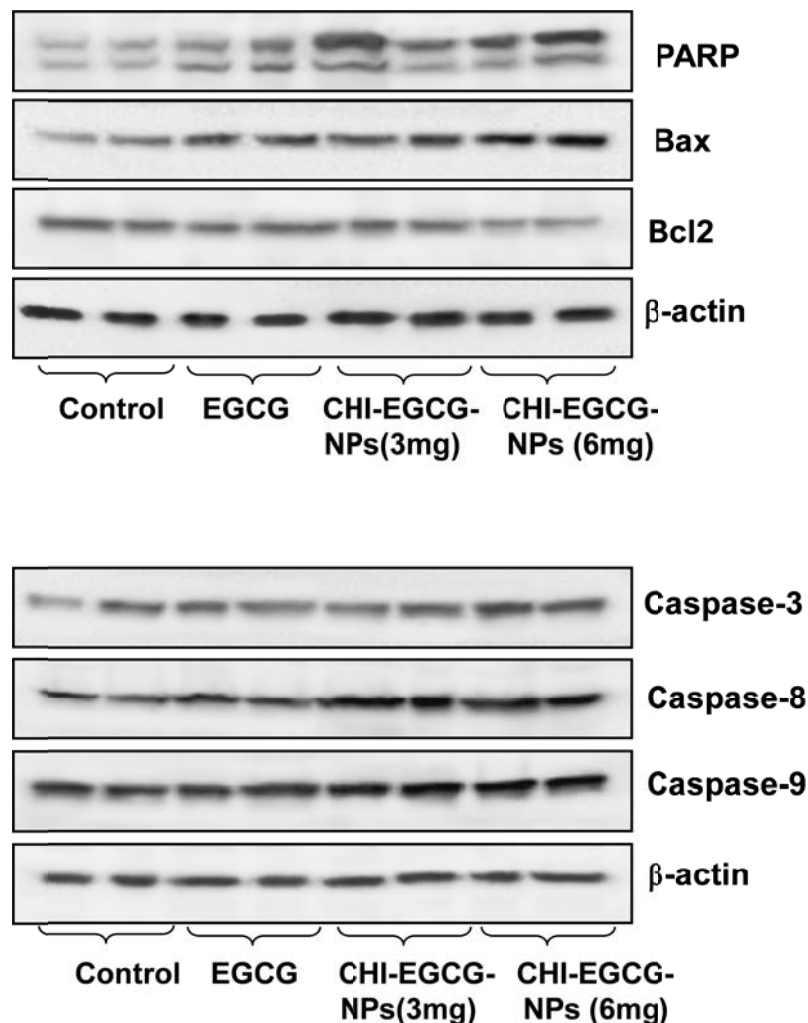


Figure 7. Effect of CHI-EGCG-NPs on the protein expression of PARP, Bax, Bcl2 and caspases-3, -8 and -9 in tumor tissues of athymic nude mice. As detailed in Figure 5, mice were treated with EGCG (40 mg/kg b.wt.), CHI-EGCG-NPs (3 mg/kg b.wt.) and CHI-EGCG-NPs (6 mg/kg b. wt.). Tissue lysate was prepared and 40 μ g protein was subjected to SDS-PAGE followed by western blot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the western blot and reprobing it for β -actin.

Effect of CHI-EGCG-NPs on cell-proliferation markers in tumor tissues of athymic nude mice

Immunohistochemical analysis: Sections (5 mm thick) were cut from paraffin embedded tumor tissues. Immunostaining was performed using specific antibodies with appropriate dilutions and was replaced with either normal host serum or block for negative controls, followed by staining with appropriate HRP-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counter stained with a weak solution of hematoxylin stain. The stained slides were dehydrated and mounted in permount and visualized on Nikon Eclipse Ti system (Nikon Instruments, Inc.). Images were captured with an attached camera linked to a computer.

Ki-67 and PCNA are well known markers of cellular proliferation. We observed that there was markedly less expression of Ki-67 and PCNA in tissues of mice treated with CHI-EGCG-NPs than group treated with EGCG and control (Figure 8).

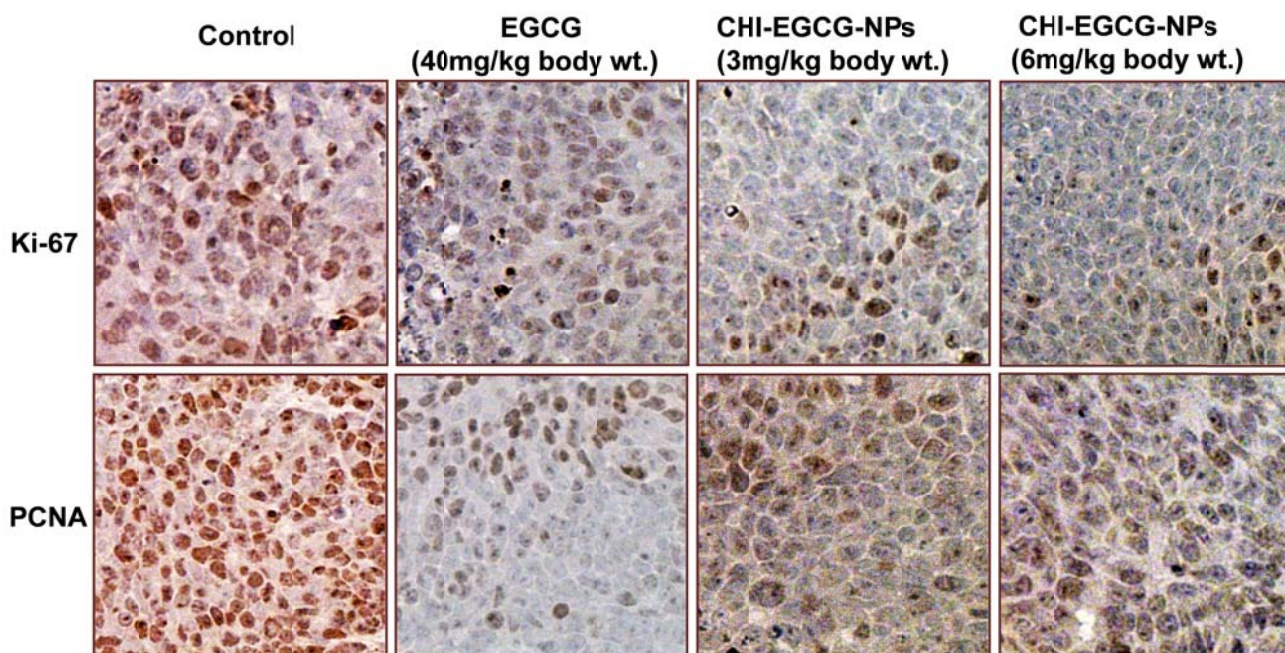


Figure 8. Effect of CHI-EGCG-NPs on cell-proliferation markers in tumor tissues of athymic nude mice. As detailed in Figure 5, mice were treated with EGCG (40 mg/kg b.wt.), CHI-EGCG-NPs (3 mg/kg b.wt.) and CHI-EGCG-NPs (6 mg/kg b. wt.). Tumor tissues were stained using Ki-67 and PCNA antibodies and counterstaining was performed with hematoxylin.

Effect of CHI-EGCG-NPs on angiogenesis markers in tumor tissues of athymic nude mice

Angiogenesis is crucial for the growth of tumors and metastasis and is strictly controlled by a highly coordinated process that is regulated by many molecules. Among them, platelet-derived endothelial cell adhesion molecule (CD31) and vascular endothelial growth factor (VEGF) are most common markers of tumor-associated angiogenesis.

By immunohistochemical analysis of the tumor tissue samples from athymic nude mice, we found more profound expression of CD31 and VEGF positive cells in control and EGCG treated groups than in CHI-EGCG-NPs treated groups (Figure 9).

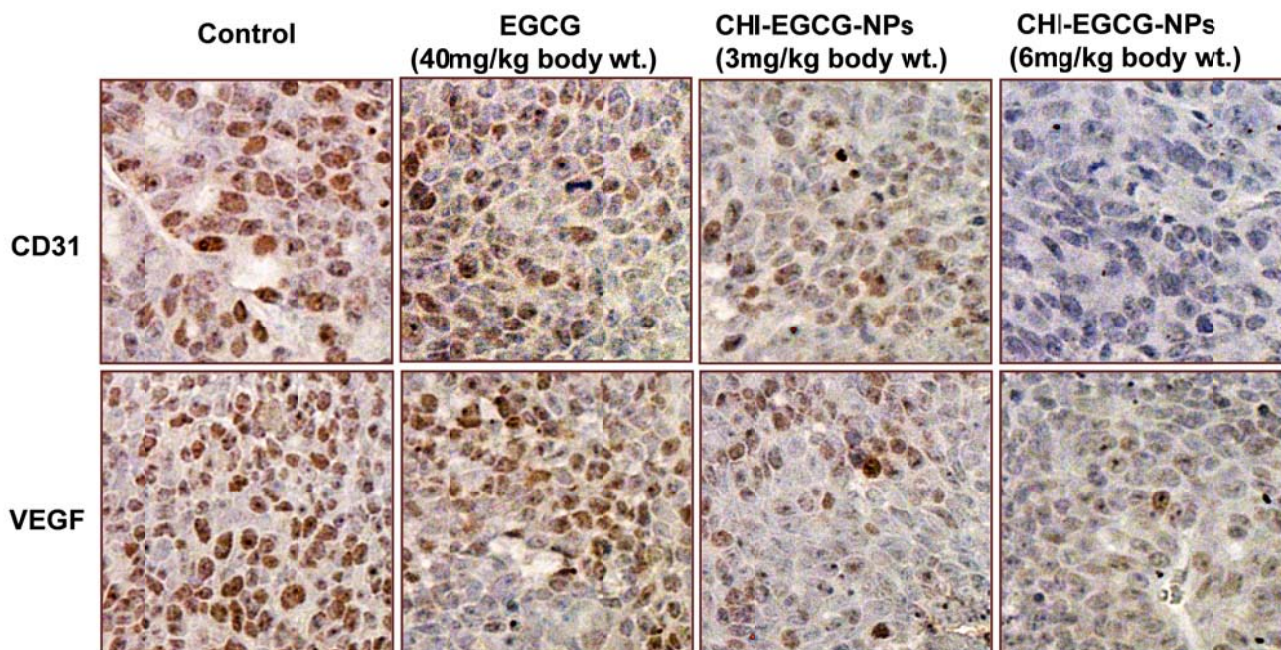


Figure 9. Effect of CHI-EGCG-NPs on angiogenesis markers in tumor tissues of athymic nude mice. As detailed in Figure 5, mice were treated with EGCG (40 mg/kg b.wt.), CHI-EGCG-NPs (3 mg/kg b.wt.) and CHI-EGCG-NPs (6 mg/kg b. wt.). Tumor tissues were stained using CD31 and VEGF antibodies and counterstaining was performed with hematoxylin.

KEY RESEARCH ACCOMPLISHMENTS:

- Prevention of cancer by natural agents is often restricted by the lack of delivery of desired levels in target tissue thus limiting their bioavailability and clinical outcome. Hence, newer approaches are required to improve bioavailability and to decrease toxicity of natural agents to accomplish maximum response. Among all natural agents, green tea has shown promise in preclinical, epidemiological and initial clinical studies, especially for PCa.
- As reported last year, CHI-EGCG-NPs were further characterized by TEM. This technique allowed us to directly visualize the CHI-EGCG-NPs. As shown in Figure 2, the nanoparticles have a size of around 200 nm; this is further supported by our earlier DS data. Additionally, from the TEM picture it is clear that the nanoparticles are spherical in shape.
- To test the stability and release kinetics of CHI-EGCG-NPs we studied the release kinetics in simulated gastric juice and simulated intestinal fluid. It is clear from the Figure 3 that the

release of EGCG from these nanoparticles is very slow in acidic medium; even at 24 hrs only ~10% of EGCG was released. On the other hand, release of intestinal fluid is much faster, and around 50% of EGCG was released in 24 hrs. Then there was a steady release of EGCG. This is strong evidence that these nanoparticles are capable of avoiding degradation in the stomach and that they do not release EGCG. And, they are capable of releasing EGCG in a neutral medium.

- Through the studies in athymic nude mice, we established the anti-tumor efficacy of oral CHI-EGCG-NPs vs. EGCG alone. Oral administration of CHI-EGCG-NPs to athymic nude mice implanted with human PCa cells resulted in:
 - Induction of apoptosis as determined by cleavage of PARP, up regulation of Bax and down regulation of Bcl2 proteins (Figure 7)
 - Induction of active caspases-3,-8 and -9 (Figure 7)
 - Inhibition of cell-proliferation markers (Figure 8)
 - Inhibition of the markers of angiogenesis (Figure 9)

ONGOING ANIMAL STUDIES:

We are currently investigating if supplementation of oral nanoEGCG will inhibit the development of PIN lesions in the Nkx3.1/Paten mutant mice via modulations in i) pro-inflammatory milieu, and ii) oxidative stress in the prostate. Further, we will determine the preventive and therapeutic efficacy of oral nanoEGCG and to identify the stage of prostate cancer development that is most vulnerable to the anti-cancer effects of oral nanoEGCG in the transgenic TRAMP mice.

REPORTABLE OUTCOMES:

Khan N, Adhami VIM, Siddiqui IA, Bareli DJ, Mouse SA and Hasan Mukhtar. Oral administration of naturally occurring chitosan based nanoformulated green tea polyphenol EGCG effectively inhibits prostate cancer cell growth in a xenograft model. The 103rd Annual Meeting of the American Association for Cancer Research, Chicago, IL, USA, Proc Amer Assoc Cancer Res, 53: 5438, 2012.

CONCLUSION:

Based on our promising data, we suggest that the concept of nano-chemoprevention (i.e. encapsulation of chemopreventive agents in nanoparticles) possesses strong merit and rationale for conducting additional detailed *in vivo* studies in appropriate animal models with relevance to human disease. As with many other nanoparticles, the advantage of using PLA-PEG nanoparticles lies in their high surface area to volume ratio, which presumably allows them to upload more EGCG while maintaining the small size. This is particularly important because the enhanced conjugation of nanoparticles with agents often results in the generation

of nanoparticles that are sparingly taken up by the diseased cells, owing to their bigger size and, thereby, undermining their medical usefulness. Also, the therapeutic/clinical importance of PLA-PEG nanoparticles relies on the fact that, being biodegradable, they rarely pose any toxicity. Through this study, we propose a novel preventive and therapeutic modality using EGCG that addresses issues related to bioavailability, that is a major reason for its limited success in humans.

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3. William WN Jr, Heymach JV, Kim ES, Lippman SM. Molecular targets for cancer chemoprevention. *Nat Rev Drug Discov* 2009; 8:213-25. PMID: 19247304.
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INTRODUCTION:

Despite extensive efforts that have been put to prevent cancer through the use of natural agents, Lack of delivery of desired levels of natural agents limits their bioavailability and poses a challenging issue for their effective clinical outcome. Therefore, in order to achieve maximum response of a natural agent, novel strategies are urgently needed to enhance the availability, effectiveness and to reduce perceived toxicity. Because most biological processes including those that are cancer-related occur at nanoscale, nanotechnology could serve as a potential tool for this unmet need. Among all natural agents green tea has shown promise in preclinical, epidemiological and initial clinical studies and limited efficacy in other human trials probably due to its poor bioavailability. In a recent proof-of-principal study, we reported significant dose-advantage of encapsulated EGCG (nanoEGCG) over non-encapsulated EGCG and advocated the novel concept of nanochemoprevention (1). However, the nanoparticles used in this study are unstable in acidic environment and therefore not recommended for oral consumption. We have been successful in developing an oral formulation of nanoEGCG employing a naturally occurring polymer chitosan, which we observed to result in a steady and sustained release of EGCG in the blood of mice following oral administration. This nano formulated green tea remains longer in the system compared to non-encapsulated EGCG and therefore is more potent. Our uniquely formulated oral nanoEGCG was synthesized in a mild acidic condition by promoting the interaction of NH₃ group present in chitosan with the phosphate group present in Adenosine 5'-tri-phosphate (ATP). Size distribution and zeta potential of chitosan based nanoparticles encapsulating EGCG was determined by using a Malvern zetasizer (Malvern Instrumentation Co, Westborough, MA). The size of the nanoparticles was found to be in the range of ~200-250 nm in diameter and zeta potential was found to be positive (+ive) as anticipated. The positive charge is an indication of superior muco-adhesive properties of the nanoformulation, which is a prerequisite for any oral formulation.

We tested the potential of our uniquely formulated nanoEGCG in transgenic adenocarcinoma of the mouse prostate (TRAMP) model of human prostate cancer and found that it resulted in significant tumor growth. We also expect that oral consumption of nanoEGCG will result in enhanced and sustained availability of EGCG leading to a robust decrease in the effective concentration and will prevent development of cancer in mouse models of prostate cancer that have relevance to human disease.

This paradigm shifting study will be an ideal strategy to jumpstart the field of chemoprevention of cancer in a new and novel way. We believe that our approach will have very significant impact considering the fact that cancer is the second most common cause of death in the United States, exceeded only by heart disease and green tea has shown promise against several cancer types. This application proposes a novel modality involving nanoencapsulation of EGCG for oral consumption designed for prevention and treatment of PCa. The immediate translational value of the work is to enhance the bioavailability and overcome associated toxicity of EGCG by reducing its effective concentration. Successful completion of our research aims should result in advancing our knowledge on the use of nanoEGCG for efficient systemic delivery, enhanced bioavailability and limited unwanted toxicity.

BODY:

TRAMP tumor study

We determined the effect of oral nanoEGCG on the growth of tumors in an *in-vivo* situation utilizing TRAMP model. The regression trial is designed to assess whether oral formulation of chitosan nanoparticles encapsulating epigallocatechin-3-gallate (Chit-nanoEGCG) has therapeutic effects on established tumors in the TRAMP model. The TRAMP mice are unique model to define preventive/therapeutic strategies against prostate cancer. Recent published studies have demonstrated the utility of this model for prostate cancer chemoprevention studies (2-5). To conserve the cost, we have established a breeding colony of TRAMP mice at our Animal Resource Facility. Male and female TRAMP mice developed in C57BL/6 background will be bred and maintained in the Animal Resource Facility at Medical Sciences Center, University of Wisconsin. We have been breeding these animals routinely by crossing pure C57BL/6 TRAMP females (transgene +ive) with pure C57BL/6 or non-transgenic TRAMP (transgene -ive) males to generate male TRAMP (transgene +ive) mice for experiments. The presence of the transgene is determined by PCR amplification of DNA extracted from the tail snips of pups at 4 weeks of age. **Study design and treatment:** We selected the animals at 24 weeks of age when the mice had established tumors. Mice were treated with EGCG (40 mg/kg body wt.) and Chit-nanoEGCG (6 mg/kg body wt., by intubation, five days a week) so as to assess its effect on regression of tumors. Five animals each from control and experimental groups were randomly selected and monitored for tumor growth by MRI at 32 weeks of age. Mice were evaluated for tumor growth at 32 weeks of age by: evaluating tumor growth in terms of tumor burden by magnetic resonance imaging, and monitoring effects on apoptosis, proliferation markers (PCNA, Ki-67) and markers of angiogenesis (CD31 and VEGF) in tumor tissues at 32 weeks.

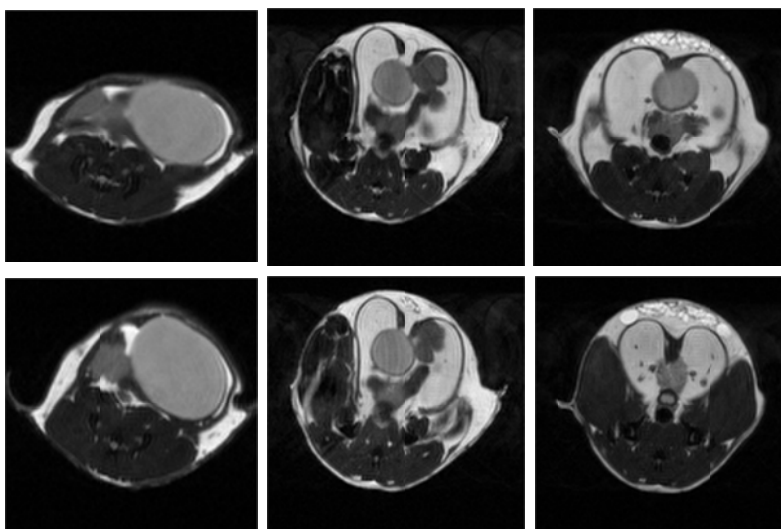


Figure 1: Representative images of MRI with interslice distance of 2 mm from Control (A), EGCG (B) and Chit-nanoEGCG supplemented mice.

A

B

C

Imaging in these animals was performed by using a whole body Varian 4.7 Tesla imager (Varian, Inc. Magnetic Resonance Systems, Palo Alto, CA) horizontal bore imaging/spectroscopy system (7.0cm ID) equipped with isoflurane gas anesthesia system. To identify prostate gland, urinary bladder was used as the reference point since the gland is located anatomically inferior and posterior to the bladder, encircling urethra. Progression of the disease was also monitored non-invasively by magnetic resonance. Prostate and tumor sizes were assessed by MRI at 32 weeks of age in the control mice and were consistent with the development and progression of prostate cancer and significantly greater than those of Chit-nanoEGCG supplemented mice. Prostate and tumor sizes in Chit-nanoEGCG-supplemented male TRAMP mice were substantially less than in the control water-fed animals. Chit-nanoEGCG-supplemented TRAMP mice exhibited a significant reduction in the development of prostate cancer measured 32 weeks on test. Because tumors were not quantifiable in Chit-nanoEGCG supplemented animals quantification of tumor volumes was not possible.

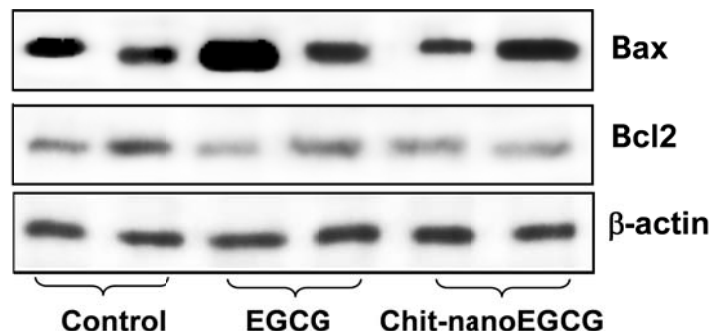
Effect of Chit-nanoEGCG on apoptosis in tumor tissues of TRAMP mice:

Protein extraction and western blotting

For western blotting, 30-50 µg protein was resolved over 8-12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% nonfat dry milk/1% Tween 20; in 20 mM TBS, pH 7.6) for 1 hr at room temperature, incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for one and half hr to overnight at 4°C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate obtained from Amersham Life Science Inc. (Arlington Height, IL, USA) and detected by chemiluminescence and autoradiography using Bio-Rad Gel-Doc (Bio-Rad Laboratories Inc., Hercules, CA).

Poly (ADP-ribose) polymerase (PARP) is involved in numerous cellular processes such as cellular proliferation, apoptosis in ischemic conditions, DNA recombination and repair and necrotic cell death. Our data in Figure 2 shows that on treatment with Chit-nanoEGCG (6 mg/kg body wt.) the full size PARP (116 KD) protein was cleaved to yield an 85 KD fragment on treatment with Chit-nanoEGCG.

Bax is a proapoptotic protein while Bcl2 is anti-apoptotic. As shown in Figure 2, treatment with Chit-nanoEGCG caused significant increase in the protein expression of Bax and decrease in the protein expression of Bcl2.



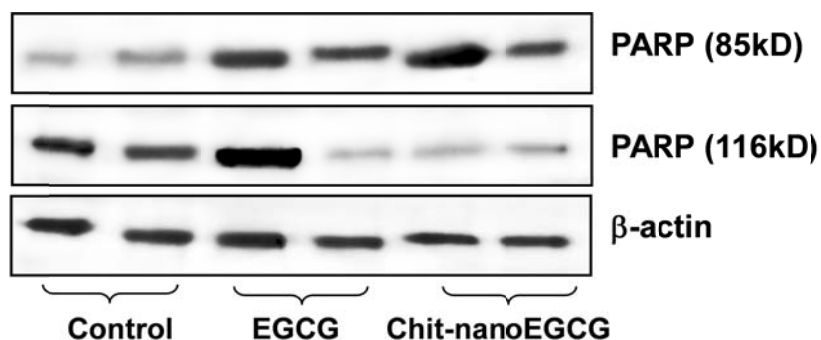


Figure 2. Effect of Chit-nanoEGCG on the protein expression of PARP, Bax, and Bcl2 in tumor tissues of TRAMP mice. Mice were treated with EGCG (40 mg/kg b.wt.) and Chit-nanoEGCG (6 mg/kg body wt.). Tissue lysate was prepared and 40 μ g protein was subjected to SDS-PAGE followed by western blot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the western blot and reprobing it for β -actin.

Effect of Chit-nanoEGCG on cell-proliferation markers in tumor tissues of TRAMP mice

Immunohistochemical analysis:

Sections (5 mm thick) were cut from paraffin embedded tumor tissues. Immunostaining was performed using specific antibodies with appropriate dilutions and was replaced with either normal host serum or block for negative controls, followed by staining with appropriate HRP-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counter stained with a weak solution of hematoxylin stain. The stained slides were dehydrated and mounted in permount and visualized on Nikon Eclipse Ti system (Nikon Instruments, Inc.). Images were captured with an attached camera linked to a computer.

Ki-67 protein is involved in regulation of cell cycle and cell proliferation and is expressed in proliferating cells during all active phases of the cell cycle and is regarded as the most promising biomarker for cell-proliferation (6). Proliferating cell nuclear antigen (PCNA) plays an important role in eukaryotic DNA synthesis and cell cycle regulation. It is considered as a cell proliferation marker and its expression is significantly increased in the process of malignant transformation of normal epithelium. We found that there was less expression of Ki-67 and PCNA in tissues of mice treated with Chit-nanoEGCG than group treated with EGCG and control (Figure 3).

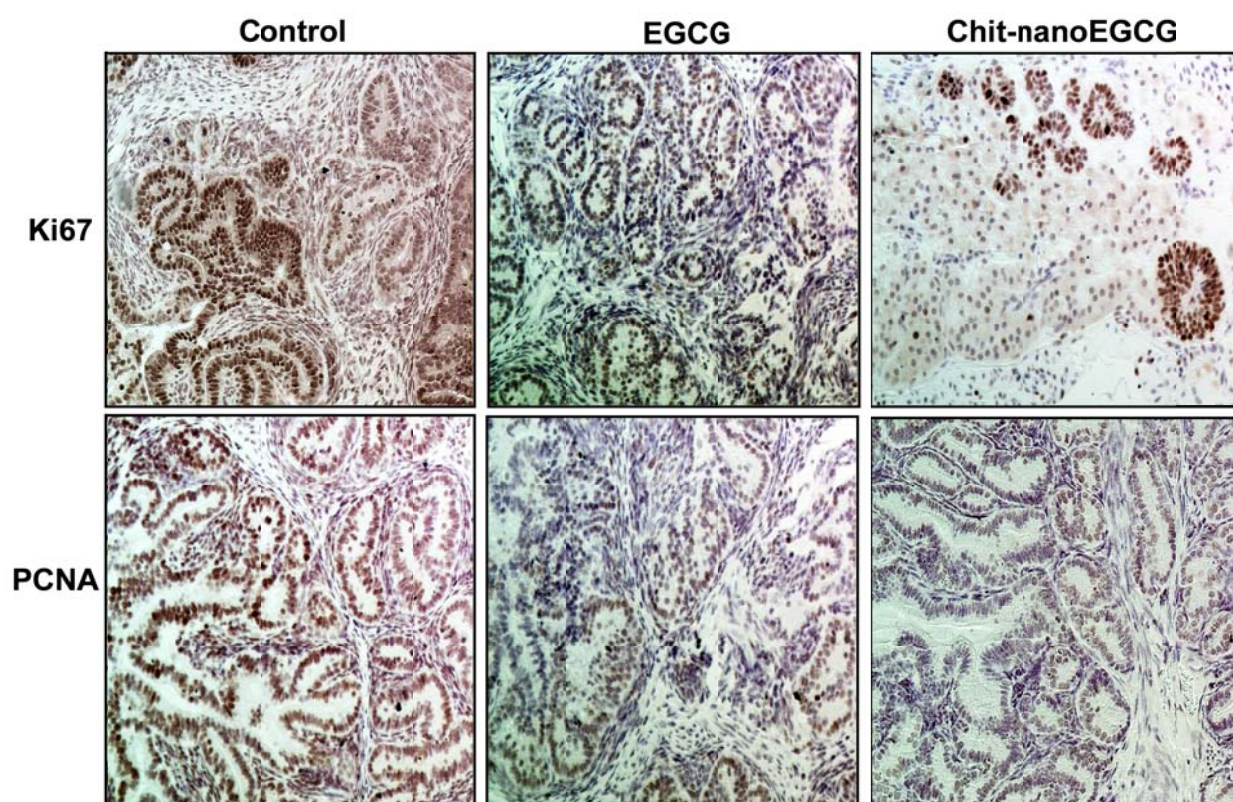


Figure 3. Effect of Chit-nanoEGCG on cell-proliferation markers in tumor tissues of TRAMP mice. Mice were treated with EGCG (40 mg/kg body wt.) and Chit-nanoEGCG (6 mg/kg body wt.). Tumor tissues were stained using Ki-67 and PCNA antibodies and counterstaining was performed with hematoxylin.

Effect of Chit-nanoEGCG on angiogenesis markers in tumor tissues of TRAMP mice

The process of angiogenesis is very critical during the development of tumors and many anti-tumor therapies target metastasis. The development of angiogenesis involves the activation, proliferation, and migration of endothelial cells toward angiogenic stimuli produced by the tumor. CD31 is a transmembrane glycoprotein and a platelet-endothelial cell adhesion molecule which recognizes pre-existing and newly formed vasculature regardless of size in normal and PCa tissues with same intensity (7). Vascular endothelial growth factor (VEGF), is one of the most vital angiogenic factor which binds to the receptor tyrosine kinases expressed on endothelial cells (8). We found more intense expression of CD31 and VEGF positive cells in control and EGCG treated groups than in groups treated with Chit-nanoEGCG by immunohistochemical analysis of the tumor tissue samples from TRAMP mice (Figure 4).

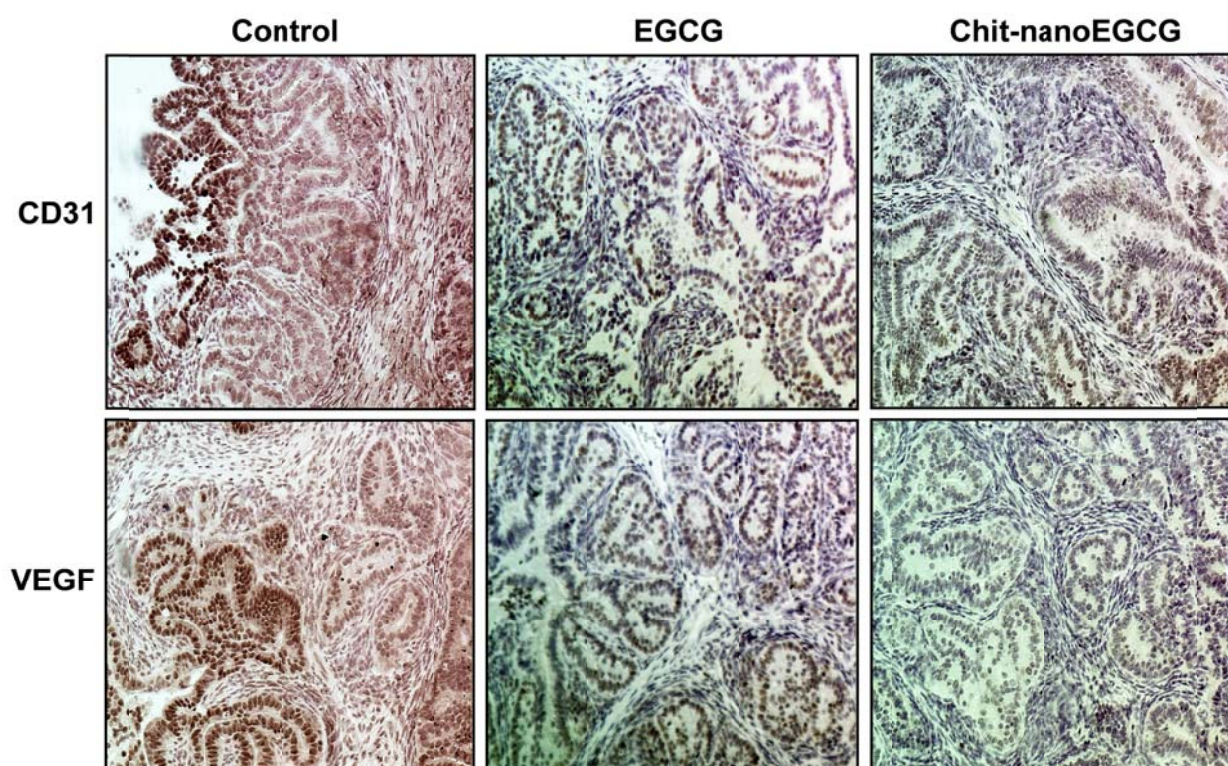


Figure 4. Effect of Chit-nanoEGCG on angiogenesis markers in tumor tissues of TRAMP mice. Mice were treated with EGCG (40 mg/kg body wt.) and Chit-nanoEGCG (6 mg/kg body wt.). Tumor tissues were stained using CD31 and VEGF antibodies and counterstaining was performed with hematoxylin.

KEY RESEARCH ACCOMPLISHMENTS:

- Through the studies in TRAMP mice, we established the anti-tumor efficacy of oral Chit-nanoEGCG vs. EGCG alone. Oral administration of Chit-nanoEGCG to TRAMP mice resulted in:
 - Prostate and tumor sizes in nanoEGCG-supplemented male TRAMP mice were substantially less than in the control water-fed animals (Figure 1).
 - Chit-nanoEGCG-supplemented TRAMP mice exhibited a significant reduction in the development of prostate cancer measured 32 weeks on test (Figure 1).
 - Induction of apoptosis as determined by cleavage of PARP, induction of Bax and suppression of Bcl2 proteins (Figure 2).
 - Inhibition of cell-proliferation markers, Ki-67 and PCNA (Figure 3).

- Inhibition of the markers of angiogenesis, CD31 and VEGF (Figure 4)

REPORTABLE OUTCOMES:

Khan N, Bharali DJ, Adhami VM, Siddiqui IA, Cui Huadong, Shabana SM, Mousa SA and Hasan Mukhtar. Oral administration of naturally occurring chitosan based nanoformulated green tea polyphenol EGCG effectively inhibits prostate cancer cell growth in a xenograft model. *Carcinogenesis* (Under Revision)

CONCLUSION:

The immediate translational value of the work, we propose, is to enhance the bioavailability and overcome associated toxicity of EGCG by reducing its effective concentration. With the proof-of-principle for superior efficacy of oral nanoEGCG for prostate cancer prevention obtained through this proposal, same technology could be extended to other potentially useful agents for other cancer sites. Thus, we believe to open a new and challenging paradigm for making cancer chemoprevention a success story. We believe that our approach will have very significant impact considering the fact that cancer is the second most common cause of death in the United States, exceeded only by heart disease and green tea has shown promise against several cancer types.

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Oral administration of naturally occurring chitosan based nanoformulated green tea polyphenol EGCG effectively inhibits prostate cancer cell growth in a xenograft model

Short Title:

Chit-nanoEGCG for prostate cancer

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Abstract:

Prevention of cancer by natural agents is often restricted by the lack of delivery of desired levels in target tissue thus limiting their bioavailability and clinical outcome. Hence, newer approaches are required to improve bioavailability and to decrease toxicity of natural agents to accomplish maximum response. Among all natural agents, green tea has shown promise in preclinical, epidemiological and initial clinical studies, especially for prostate cancer (PCa). Much of the effects of green tea are considered to be exerted by its major polyphenol (-) epigallocatechin-3-gallate (EGCG). We reported significant dose-advantage of polylactic acid-polyethylene glycol (PLA-PEG) encapsulated EGCG (nanoEGCG) over non-encapsulated EGCG (*Cancer Res.* 2009; 69: 1712-1716), for PCa in cell-culture and xenograft model. However, PLA-PEG is unstable in acidic environment and is therefore not recommended for oral consumption, a desired route for chemopreventive agents. We developed a formulation of nanoEGCG suitable for oral delivery employing a naturally occurring polymer chitosan, which here is referred to as Chit-nanoEGCG. This Chit-nanoEGCG when administered to mice resulted in a steady and sustained release of EGCG in the plasma of mice at 1 and 8 hours post-treatment. The effect of Oral Chit-nanoEGCG was then determined on the growth of tumors in athymic nude mice implanted with CWR22R?1 cells. Treatment with Chit-nanoEGCG (3 and 6 mg/kg body wt.) resulted in significant inhibition of tumor growth. At these doses, non-encapsulated EGCG was totally ineffective. In control group, the average tumor volume of 1,200 mm³ was reached in 32 days after tumor cell inoculation. At this time point, the average tumor volume was 514, 310 and 216 in non-encapsulated EGCG, Chit-nanoEGCG (3mg/kg body wt.) and Chit-nanoEGCG (6mg/kg body wt.) treated groups, respectively. The average tumor volume of 1,200 mm³ was achieved on 46, 53 and 60 days after tumor cell inoculation in these three groups, respectively. There was significant inhibition of secreted prostate specific antigen (PSA) levels by 13-36%, 26-54% and 57-72% in EGCG, Chit-nanoEGCG (3mg/kg body wt.) and Chit-nanoEGCG (6mg/kg body wt.) treated groups, respectively as compared to the control group. In tumor tissues of mice treated with both doses of Chit-nanoEGCG as compared to group treated with EGCG and controls, there was significant (i) reduction in Ki-67 and proliferating cell nuclear antigen (PCNA) (ii) induction of poly (ADP-ribose) polymerases (PARP) cleavage, (iii) activation of caspases and, (iv) increase in the protein expression of Bax and decrease in Bcl2. Through this study, we propose a novel preventive and therapeutic modality using EGCG that addresses issues related to bioavailability, that is a major reason for its limited success in humans.

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Oral administration of naturally occurring chitosan based nanoformulated green tea polyphenol EGCG effectively inhibits prostate cancer cell growth in a xenograft model

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Running title: Nanoformulated EGCG for prostate cancer

Abstract

In preclinical animal models, several phytochemicals have shown excellent potential to be used as effective agents in preventing and treating many cancers. However, their poor bioavailability and half-life pose a major challenge for their usefulness for human consumption. To overcome this limitation, we recently introduced the concept of nanochemoprevention by encapsulating useful bioactive food components for their slow release to enhance their bioavailability. Here we report the synthesis, characterization, and efficacy assessment of a nanotechnology-based oral formulation of chitosan nanoparticles encapsulating epigallocatechin-3-gallate (Chit-nanoEGCG) for the treatment of prostate cancer (PCa) in a preclinical setting. Chit-nanoEGCG with a size of <200 nm diameter and encapsulating EGCG as determined by Dynamic Light Scattering and Transmission Electron Microscope showed slow release of EGCG in simulated gastric juice acidic pH and faster release in simulated intestinal fluid. The antitumor efficacy of Chit-nanoEGCG was assessed in subcutaneously implanted 22Rv1 tumor xenografts in athymic nude mice. Treatment with Chit-nanoEGCG resulted in significant inhibition of tumor growth and secreted prostate specific antigen levels as compared to EGCG and control groups. In tumor tissues of mice treated with Chit-nanoEGCG, as compared to groups treated with EGCG and controls, there was significant (i) induction of poly (ADP-ribose) polymerases cleavage, (ii) increase in the protein expression of Bax with concomitant decrease in Bcl2, (iii) activation of caspases and, (iv) reduction in Ki-67 and proliferating cell nuclear antigen. Through this study, we propose a novel preventive and therapeutic modality for PCa using EGCG that addresses issues related to bioavailability.

Introduction

Although extensive efforts have been put to prevent and treat cancer through the use of bioactive food components and other agents, lack of delivery of desired levels thus limiting their bioavailability, remains an obstacle for effective clinical outcome (1-3). Therefore, it is essential to find new strategies to deliver the bioactive food components to the human population for the prevention and treatment of cancer. For a natural agent to elicit maximum response, newer approaches are required for increasing the bioavailability and reducing the toxicity after continuous use for extended period of time. Nanotechnology can be useful against cancer as many of the related processes occur at nano-scale. (4,5). This delivery system could overcome many biological, biophysical and biomedical barriers (4,6).

An oral route is the most accepted way of drug administration in terms of patient compliance and convenience. However, to date oral delivery of many drugs, in particular of many of the active ingredients with high therapeutic value in natural products has proven to provide poor bioavailability when administered orally (7-9). The main obstacle that hinders the clinical availability of naturally derived compounds is the harsh condition of the gastrointestinal tract, mainly due to physical barriers like the intestinal epithelium and degradation by various enzymes and gastric juices (10,11). Epigallocatechin-3-gallate (EGCG), the natural active ingredient of green tea, is the most widely used natural product and has shown excellent potential in several studies to treat and prevent many cancers including PCa (12-18). It is imperative to look for a carrier system that can protect the natural active ingredient from environmental assault and that has the capacity to improve the bioavailability. Utilization of ultramodern nanotechnology to incorporate natural active ingredients for the development of aqueous nanoformulations for oral delivery might be an answer to many of the inherent bioavailability problems.

In our earlier study while introducing the concept of nanochemoprevention, we reported significant dose-advantage of polylactic acid-polyethylene glycol (PLA-PEG) encapsulated EGCG (nanoEGCG) over non-encapsulated EGCG. NanoEGCG had over ten-fold dose advantage for exerting its pro-apoptotic and anti-angiogenic effects (15). However, this custom-made nanoparticle formulation was designed for systemic delivery, and due to its non-mucoadhesive properties, negative surface charge, non-stimuli sensitive behavior, and relatively larger size, were unsuitable for oral administration (15). To overcome these problems, we have chosen chitosan nanoparticles for oral delivery of EGCG (Chit-nanoEGCG). We synthesized nanoparticles made up of the natural biopolymer chitosan and incorporated EGCG, which appeared to be stable in the acidic environment of the stomach and prevented release of EGCG in the stomach. Chitosan nanoparticles have been largely used for efficient oral delivery of numerous drugs due to their characteristic mucoadhesive properties (19-22). Chitosan is a natural linear polysaccharide derived by deacetylation of chitin from shrimp/crabs, insects, and fungi, and is also known for its non-toxic, non-immunogenic properties (23). It has already been used as a pharmaceutical excipient, a weight loss supplement, an experimental mucosal adjuvant, and an FDA-approved hemostatic dressing (24-27). It has a positive charge due to the presence of ample of NH_2 groups that also provide the necessary properties to adhere to the gastrointestinal tract for a longer time. Thus, chitosan nanoparticles' increased retention time, ultrafine size, and ability to release the drug for a longer time make it an ideal oral delivery vehicle.

In this study, we report the synthesis and characterization of chitosan nanoparticles with a size in the range of 150-200 nm diameter encapsulating EGCG. These nanoparticles showed a slow release of EGCG in acidic pH (simulated gastric juice) and faster release in simulated intestinal fluid (neutral pH). The antitumor efficacy of this uniquely formulated Chit-nanoEGCG was

assessed in subcutaneously implanted tumor xenografts in athymic nude mice. Significant improvement of therapeutic benefit against PCa tumors was observed with this Chit-nanoEGCG compared to its free counterpart.

Material and Methods

Materials

Anti- Bax, Bcl2, active caspases-3, 8 and 9, PARP antibodies were obtained from Cell Signaling Technology (Beverly, MA). VEGF antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Ki-67 and CD31 antibodies were obtained from Abcam (Cambridge, MA). Human PSA Elisa kit was procured from Anogen (Mississauga, Ontario). Anti-mouse and anti-rabbit secondary antibody horseradish peroxidase conjugate was obtained from Amersham Life Science Inc. (Arlington Height, IL). BCA Protein assay kit was obtained from Pierce (Rockford, IL). Novex precast Tris-glycine gels were from Invitrogen (Carlsbad, CA). EGCG, Pentasodium tripolyphosphate hexahydrate (TPP), heptafluorobutyric acid (HFBA), formaldehyde, acetonitrile, and cellulose dialysis tubing were purchased from Sigma Aldrich Co. (St. Louis, MO). Water soluble chitosan was obtained from Polysciences Inc. (Warrington, PA).

Synthesis of chitosan nanoparticles encapsulating EGCG (Chit-nanoEGCG)

To synthesize chitosan nanoparticles, 45 ml of a solution of EGCG (10 mg/ml in deionized water) was added to 5 ml of 1% water-soluble chitosan and stirred for 1 h. Next, 1 ml of a pentasodium tripolyphosphate hexahydrate (TPP) (10 mg/ml in deionized water) was added drop by drop, with constant stirring. The entire solution was then sonicated for about 30 seconds using a probe sonicator, and allowed to stir for approximately 4 h. This solution containing EGCG nanoparticles was dialyzed to remove the impurities and the free EGCG using a 100 KD cut-off dialysis membrane. The solution was then lyophilized to get the nanoformulation in powdered form and redispersed in deionized water for further use.

Size measurement by Dynamic Light Scattering (DLS)

Size distribution of Chit-nanoEGCG in aqueous dispersion was determined by using a Malvern zeta sizer (Malvern Instrumentation Co., Westborough, MA) and was compared with the Chit-nano without incorporation of EGCG (void nanoparticles). Two ml of Chit-nanoEGCG/ void nanoparticles were placed in a 4-sided, clear, plastic cuvette and analyzed directly at 25°C. The size of the nanoparticles was found to be less than 200 nm in diameter.

Size measurement by Transmission Electron Microscope

The size and morphology of Chit-nanoEGCG nanoparticles were examined by transmission electron microscopy (TEM) using a JEOL JEM-100CX TEM (JEOL, USA, Inc., Peabody, MA). One drop of the Chit-nanoEGCG solution was mounted on a thin film of amorphous carbon deposited on a copper grid (300 mesh). The solution was air dried, and the sample was examined directly under the microscope.

Determination of entrapment efficiency (loading efficiency)

The amount of EGCG was determined by disintegrating the nanoparticles and measuring the EGCG by LC/MS. This also helps in determination of the entrapment efficiency of EGCG in the nanoformulation. The redispersed nanoparticles were disintegrated by adding an acetic acid solution (by incubating it in 1% v/v solution for 30 min). The entire solution was passed through a filter Millipore centrifugal device of 100 KD cut-off with the help of centrifugation, at around 6500 rpm for 15 minutes to separate the EGCG. The concentration of the centrifugate containing EGCG was determined using LC/MS. The entrapment efficiency was determined by the following formula:

$$\text{Entrapment efficiency (loading)} = ([\text{EGCG}]_f) / ([\text{EGCG}]_t) \times 100$$

where, $[\text{EGCG}]_f$ is the concentration of EGCG in the centrifugate and $[\text{EGCG}]_t$ is the theoretical concentration of EGCG (meaning total amount of EGCG added initially).

Release kinetics studies

The cumulative release of EGCG from Chit-nanoEGCG nanoparticles was studied in simulated gastric juice and simulated intestinal fluid. For this cumulative release kinetic study, a known amount of nanoparticles encapsulated EGCG was suspended in 15 ml of simulated gastric juice/simulated intestinal juice. The solution was kept in an incubator at room temperature (25°C). At predetermined time intervals, 500 µl of the solution was filtered through Millipore Centricon tubes containing a 100 KD membrane to separate released EGCG from the nanoparticles. The amount of released EGCG present in the filtrate was determined by LC/MS.

Protein extraction and immunoblotting

The tumor tissue samples were homogenized in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4 , 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, pH 7.4) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA). The homogenate was then centrifuged at 14 000 g for 25 min at 4°C, and the supernatant (whole cell lysate) was collected, aliquoted and stored at -80°C.

For immunoblotting, 30-50 µg protein was resolved over 8-12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% non-fat dry milk/1% Tween 20; in 20 mM TBS, pH 7.6) for 1 h at room temperature, incubated with

appropriate monoclonal or polyclonal primary antibody in blocking buffer for one and half h to overnight at 4°C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate obtained from Amersham Life Science Inc. (Arlington Height, IL, USA) and detected by chemiluminescence and autoradiography using Bio-Rad Gel-Doc (Bio-Rad Laboratories Inc., Hercules, CA).

In-vivo tumor xenograft in athymic nude mice

Ethics Statement: The studies were conducted according to the Institutional guidelines for the care and use of animals and were approved by Animal Care and Use Committee, School of Medicine and Public Health, University of Wisconsin-Madison.

The effects of oral Chit-nanoEGCG were studied *in-vivo* using athymic nude mice xenograft. Athymic (*nu/nu*) male nude mice were obtained from NxGen Biosciences (San Diego, CA) and housed under pathogen-free conditions with a 12-h light/12-h dark schedule, and fed with an autoclaved diet *ad libitum*. Forty eight male athymic nude mice (4 weeks of age) were housed four/cage and fed *ad libitum* with autoclaved semi-purified, AIN-76 B-40 diet. A total of 1 million 22Rv1 cells (in 50 µl DMEM + 50 µl Matrigel) were implanted by a sub-cutaneous injection on left and right sides, below the shoulders (2 tumors per mouse). Mice implanted with cells were randomly distributed into four groups of twelve each. Group I received void chitosan nano particles and served as the control. Group II received EGCG (40 mg/kg body wt). Group III received Chit-nanoEGCG (3 mg/kg body wt) and Group IV received Chit-nanoEGCG (6 mg/kg body wt). Treatments were by oral intubation and started one day post cell inoculation, 5 times a week (Monday-Friday) until tumors reached a targeted volume of 1200 mm³. The tumor size was measured by determining two perpendicular dimensions with calipers, and the volume was

calculated using the formula $(a \times b^2)/2$, where a is the longer and b is the smaller dimension. The animals were also be evaluated for body weight, consumption of food and apparent signs of toxicity. At weekly intervals phlebotomy was performed to obtain sera for PSA estimation by ELISA.

PSA estimation by ELISA

The human PSA ELISA kit from Anogen (Mississauga, Ontario) was used for the quantitative determination of PSA levels in serum.

Immunohistochemical analysis

Sections (5 mm thick) were cut from paraffin embedded tumor tissues. Immunostaining was performed using specific antibodies with appropriate dilutions and was replaced with either normal host serum or block for negative controls, followed by staining with appropriate HRP-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counter stained with a weak solution of hematoxylin stain. The stained slides were dehydrated and mounted in permount and visualized on Nikon Eclipse Ti system (Nikon Instruments, Inc.). Images were captured with an attached camera linked to a computer.

Results

Synthesis and characterization of Chit-nanoEGCG

A representative diagram comparing the size and size distribution of void chitosan nanoparticles and Chit-nanoEGCG by DLS is shown in Figure 1. It was observed that the size distribution of both void and Chit-nanoEGCG nanoparticles were around 150-<200 nm in diameter. The size and morphology was further confirmed by TEM as shown in Figure 2A, which also supported the fact that the nanoparticles had a size of <200 nm in diameter as evidenced by DLS in Figure 1. Additionally, from the TEM picture it was clear that the nanoparticles were spherical in shape (Figure 2A).

We also developed a method to determine the amount of EGCG encapsulated in the nanoparticles by LC/MS. A graphical representation comparing the release profiles in simulated gastric juice and simulated intestinal fluid is shown in Figure 2B. In the case of oral delivery of an active biomaterial/natural product the degradation starts in the stomach, due to the acidic pH. One of the most important factors to be kept in mind for carrier-mediated drug delivery through an oral route is that the carrier system must not degrade/release at acidic pH, releasing the entire drug. At the same time, it has to retain its capability to release the drug at neutral pH. To test the stability and release kinetics of Chit-nanoEGCG nanoparticles, we studied the release kinetics in simulated gastric juice and simulated intestinal fluid. It is clear from the Figure 2B that the release of EGCG from these nanoparticles is very slow in acidic medium; even at 24 h only ~10% of EGCG was released. On the other hand, release of intestinal fluid is much faster, and around 50% of EGCG was released in 24 hrs. Then there was a steady release of EGCG. This is strong evidence that these nanoparticles are capable of avoiding degradation in the stomach, they do not release EGCG and they are capable of releasing EGCG in a neutral medium.

Inhibition of the growth of human prostate carcinoma 22Rv1 cells in athymic nude mice by Chit-nanoEGCG

The treatment of athymic nude mice with nanoEGCG resulted in inhibition of AR-positive CWR22Rv1 tumor xenograft growth. The appearance of small solid tumors was observed in animals of control group 11 days after cell inoculation. This latency period was prolonged to 18 days in animals receiving EGCG (40 mg/kg body wt.) and 25 days in animals receiving Chit-nanoEGCG (3 and 6 mg/kg body wt.). There was significant reduction in growth of prostate tumors in EGCG and Chit-nanoEGCG -treated animals as compared to control group (Figures 3A-C). As depicted in Figure 3B, tumor growth as inferred by computed tumor volume, was significantly inhibited in mice receiving EGCG and Chit-nanoEGCG. In control group, the average tumor volume of 1,200 mm³ was reached in 32 days after tumor cell inoculation. At this time point, the average tumor volume was 514, 310 and 216 in EGCG, Chit-nanoEGCG (3mg/kg body wt.) and Chit-nanoEGCG (6mg/kg body wt.) treated groups, respectively. The average tumor volume of 1,200 mm³ was achieved 46, 53 and 60 days after tumor cell inoculation in EGCG, Chit-nanoEGCG (3mg/kg body wt.) and Chit-nanoEGCG (6mg/kg body wt.) treated groups, respectively (Figures 3B and C). There was dose-dependent inhibition of tumor growth by Chit-nanoEGCG and it was found to be more effective than EGCG.

Inhibition of PSA secretion in athymic nude mice by Chit-nanoEGCG

During the course of tumor growth in animals at day 2, 8, 14, 20 and 26 after inoculation, blood was collected through the mandibular bleed. Quantitative sandwich ELISA was used to determine circulating PSA levels in mouse serum secreted by 22Rv1 tumor xenografts. PSA is a serine protease with highly prostate-specific expression and is the most widely employed marker

in the detection of early PCa. For these reasons, it is considered that agents which could reduce PSA levels may have important clinical implications for PCa. It is currently the most accepted marker for assessment of PCa progression in humans and is being detected in the serum of patients with prostate diseases including prostatitis, benign prostatic hypertrophy, and PCa (28). In our study, we found that there was significant inhibition of secreted PSA levels by 13-36%, 26-54% and 57-72% in EGCG, Chit-nanoEGCG (3mg/kg body wt.) and Chit-nanoEGCG (6mg/kg body wt.) treated groups, respectively as compared to the control group normalized to tumor volume (Figure 3D). Hence, our results show that treatment of mice with Chit-nanoEGCG caused dose-dependent significant decrease in the serum PSA in athymic nude mice and the effect was more pronounced with Chit-nanoEGCG than EGCG (Figure 3D).

Cleavage of PARP, effect on Bax, Bcl2 and induction of active caspases-3, -8 and -9 by Chit-nanoEGCG

Poly (ADP-ribose) polymerase (PARP) is a nuclear protein which gets activated upon damage to DNA and modify DNA-associated proteins by adding chains of ADP ribose units (29). As shown by immunoblots in Figure 5, PARP cleavage analysis showed that the full size PARP (116 KD) protein was cleaved to yield an 85 KD fragment on treatment with Chit-nanoEGCG (Figure 4A). The mitochondrial pathway of apoptosis is activated by numerous intracellular and extracellular stress signals which result in initiation of pro-apoptotic proteins such as Bax and anti-apoptotic proteins such as Bcl2 (30). There was also a significant increase in the protein expression of Bax whereas the protein expression of Bcl-2 was significantly decreased by Chit-nanoEGCG in a dose-dependent fashion (Figure 4A). Caspases are a family of cysteine proteases which at their active site contain cysteine residue and cleave their substrate at position next to aspartate residue

and are involved in various functions such as, inflammatory, development and apoptotic pathways (31). We also found the activation of casapse-3, -8 and -9 in tumor tissues of mice treated with both doses of Chit-nanoEGCG as compared to group treated with EGCG and control (Figure 4B).

Inhibition of cell-proliferation markers in tumor tissues of athymic nude mice by Chit-nanoEGCG

Ki-67 protein is involved in regulation of cell cycle and cell proliferation. It is expressed in proliferating cells during all active phases of the cell cycle and is regarded as the most promising biomarker for cell-proliferation (32). Proliferating cell nuclear antigen (PCNA) is involved in eukaryotic DNA synthesis and plays an important role in the cell cycle regulation. Its expression is significantly increased in the process of malignant transformation of normal epithelium and is considered as a cell proliferation marker. We observed that there was markedly less expression of Ki-67 and PCNA in tissues of mice treated with Chit-nanoEGCG than group treated with EGCG and control (Figure 5).

Inhibition of angiogenesis markers in tumor tissues of athymic nude mice by Chit-nanoEGCG

Angiogenesis is a critical process during tumor development and metastasis is the target of many anti-tumor therapies (33). The process of angiogenesis involves the activation, proliferation, and migration of endothelial cells toward angiogenic stimuli produced by the tumor. CD31 is a transmembrane glycoprotein and a platelet-endothelial cell adhesion molecule which recognizes pre-existing and newly formed vasculature regardless of size in normal and PCa tissues with same intensity (34). Vascular endothelial growth factor (VEGF), is one of the most essential

angiogenic factor which exerts its biological effects by binding to its receptor tyrosine kinases, expressed on endothelial cells (35). By immunohistochemical analysis of the tumor tissue samples from athymic nude mice, we found more profound expression of CD31 and VEGF positive cells in control and EGCG treated groups than in Chit-nanoEGCG-treated groups (Figure 6).

Discussion

Green tea has shown potential in laboratory, clinical and epidemiological studies, however, concerns related to bioavailability of its most active component EGCG and perceived toxicity associated with its long-term use affect its clinical outcome (36,37). This study suggests a different approach involving nanoencapsulation of EGCG for oral consumption for the prevention and treatment of PCa. Oral consumption is the most preferred and suitable form of delivery of chemopreventive agents. Though, one disadvantage of using PLA-PEG nanoparticles is their unstable nature in acidic environment and therefore is not recommended for oral consumption (38). To overcome this obstacle we have been successful in developing an oral formulation of nanoEGCG employing a naturally occurring polymer chitosan which we observed to result in a steady and sustained release of EGCG in the plasma of mice. Chitosan nanoparticles were synthesized in aqueous conditions by promoting the interaction of the NH_2 group present in chitosan with the phosphate group present in tripolyphosphate (TPP). We chose water-soluble chitosan as opposed to the more widely used chitosan that is soluble in mildly acidic pH because using water-soluble chitosan eliminates harsh acidic conditions, which might have adverse effects on the active ingredients. This nanoformulation also has the potential to be used as a carrier system for many of the bio-active compounds that have sensitivity to acidic pH. The size of the nanoparticles can be manipulated by changing parameters like the concentration of chitosan and TPP. However, after extensive experimental studies, we found that this formulation was the optimum formulation with the highest loading efficiency of EGCG. Therefore, we chose this particular method of preparing Chit-nanoEGCG, which can produce a size around 150-200 nm with spherical morphology and with loading efficiency of around 10% w/w to the nanomaterials.

One of the major problems that hinders the use of many natural active ingredients as anticancer drugs (especially EGCG) is the poor absorption in the gastrointestinal tract. In the case of oral delivery of an active biomaterial or natural product, the degradation starts in the stomach, due to the acidic pH. Our chitosan nanoparticles provide a platform for EGCG to be embedded in the polymeric network, and therefore EGCG can be protected from degradation in the harsh conditions of the stomach and the gastrointestinal tract. One of the most important factors for carrier-mediated delivery of drugs through an oral route is that the carrier system must not degrade and release at acidic pH, thus releasing the entire drug. At the same time it has to retain its capability to release the drug at neutral pH. Our release kinetics experiments strongly support that the release of EGCG in simulated gastric juice was minimal, and at the same time EGCG was able to be released in a faster way. An initial burst at around 20% is because of the release of the desorbed EGCG from the particle surface, and thereafter the slow release up to 24 h (~50%) resulted from the diffusion of the drug to the aqueous environment, and finally the slow and steady sustained release was because of the diffusion as well as the disintegration of the polymeric nano-particulate network. The increased EGCG level observed in the blood serum after 3 h compared to free EGCG was perhaps because of the special muco-adesive properties of the chitosan polymers. Thus, it is postulated that chitosan nanoparticles can increase the retention time of the EGCG by adhering to the mucosal surface in the gastrointestinal tract as well as transiently opening the tight epithelial cellular junctions (39) and thereby increasing the level of EGCG in the blood serum.

The ultimate goal of our nanoformulation Chit-nanoEGCG was to improve the efficacy of EGCG in PCa tumor xenografts. The choice of androgen-refractory 22Rv1 cells was because of their unique properties such as rapid and reproducible tumor growth *in-vivo* and secretion of PSA

in the bloodstream of the host. Our data clearly established that Chit-nanoEGCG substantially inhibits the growth of PCa even with a 6-fold lower dose of EGCG compared to free EGCG. PSA is a serine protease with highly prostate specific expression and is the most widely employed marker in the detection of early PCa. For these reasons, agents that could reduce PSA levels may have important clinical implications for PCa. It is currently the most accepted marker for assessment of PCa progression in humans and is being detected in the serum of patients with prostate diseases including prostatitis and benign prostatic hypertrophy. Our results showed that treatment of athymic nude mice with Chit-nanoEGCG caused a dose-dependent significant decrease in the serum PSA and the effect was more pronounced with Chit-nanoEGCG than with free EGCG.

PARP is involved in several cellular activities such as regulation of transcription and DNA repair (40) and its inhibitors are being investigated in various clinical trials (29,41). The process of apoptosis comprises of the sequential activation of caspases, which are the key players in apoptotic cell death. Our results showed that there was cleavage of PARP, induction of Bax inhibition of Bcl2 and activation of caspases-3, -8 and -9, in the tumor tissues of mice treated with Chit-nanoEGCG (Fig. 5A). There was also decrease in the expression of Ki-67, PCNA (cell-proliferation marker), CD31 and VEGF (markers of angiogenesis) in tissues of mice treated with Chit-nanoEGCG than free EGCG. Although our experiments were performed in a preclinical setting, our results indicate that in the near future nanotechnology-based, non-invasive platform has tremendous potential to replace many of the invasive techniques (like radiation therapy or surgery) to treat PCa.

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Conflict of Interest Statement: None

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Figures Legends

Fig. 1. Size and size distribution measurement by Dynamic Light Scattering (DLS) of (A) chitosan-based nanoparticles encapsulating EGCG (Chit-nanoEGCG), and (B) void chitosan nanoparticles.

Fig. 2 (A). Transmission Electron Microscopy images showing the spherical size and morphology of Chit-nanoEGCG nanoparticles. **(B).** Cumulative release kinetics of EGCG from Chit-nanoEGCG in simulated gastric juice and simulated intestinal fluid.

Fig. 3. Effect of Chit-nanoEGCG on PCa tumor growth and serum PSA in athymic nude mice. Forty eight athymic nude mice were randomly divided into four groups of twelve mice in each group. Approximately one million 22Rv1 cells were s.c. injected in each flank of mouse to initiate tumor growth. One day after cell implantation, group I received void chitosan nanoparticles and served as the control. Group II received EGCG (40 mg/kg body wt). Group III received Chit-nanoEGCG (3 mg/kg body wt) and Group IV received Chit-nanoEGCG (6 mg/kg body wt) by oral intubation 5 times a week. Once tumors started to grow, their sizes were measured weekly and the tumor volume was calculated. **(A)** Photographs of excised tumors from each group at the end of study. **(B)** Average tumor volume of control group, EGCG-treated and Chit-nanoEGCG-treated mice plotted over days after tumor cell inoculation. **(C)** Number of mice remaining with tumor volumes of $1,200 \text{ mm}^3$ after they received treatment with EGCG and Chit-nanoEGCG for the indicated days. **(D)** Serum PSA levels were analyzed by ELISA. Details are described in "Materials and Methods" section.

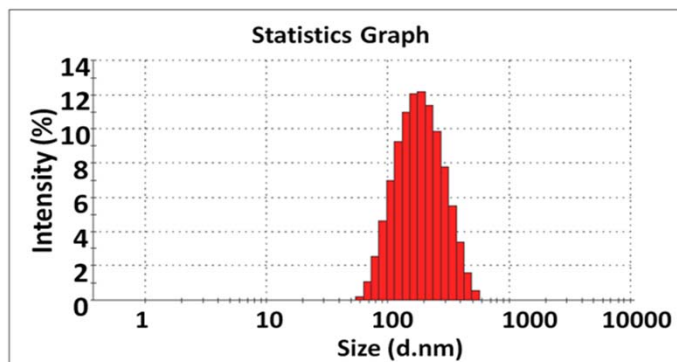
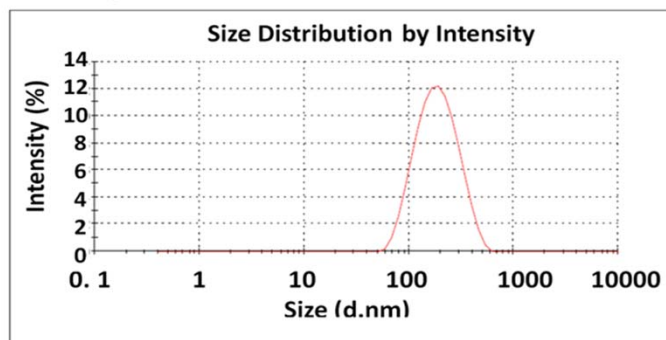
Fig. 4. Effect of Chit-nanoEGCG on PARP, Bax, Bcl2 and caspases in tumor tissues of athymic nude mice. (A) Effect of Chit-nanoEGCG on cleavage of PARP, Bax and Bcl2 in tumor tissues. (B) Effect of Chit-nanoEGCG on protein expression of caspases in tumor tissues. As detailed in “Materials and Methods”, total cell lysates were prepared and 40 µg protein was subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-actin.

Fig. 5. Effect of Chit-nanoEGCG on expression of Ki-67 and PCNA in tumor tissues of athymic nude mice. Tumor sections from athymic nude mice were stained using specific antibodies as detailed in “Materials and Methods”. Counterstaining was performed with hematoxylin. Scale bar, 50 µm. Photomicrographs (magnification, X20) show representative pictures from two independent samples.

Fig. 6. Effect of Chit-nanoEGCG on expression of CD31 and VEGF in tumor tissues of athymic nude mice. Tumor sections from athymic nude mice were stained using specific antibodies as detailed in “Materials and Methods”. Counterstaining was performed with hematoxylin. Scale bar, 50 µm. Photomicrographs (magnification, X20) show representative pictures from two independent samples.

Result (A)

	Diam. (nm)	% Intensity	Width (nm)
Z-Average(d.nm): 158	Peak1: 200nm	100	89.5
PdI:0.241	Peak2: 0.0 nm	0.0	0.00
Intercept: 0.947	Peak3: 0.0nm	0.0	0.00



Result (B)

	Diam. (nm)	% Intensity	Width (nm)
Z-Average(d.nm): 188	Peak1: 207nm	100	61.3
PdI:0.091	Peak2: 0.0 nm	0.0	0.00
Intercept: 0.975	Peak3: 0.0nm	0.0	0.00

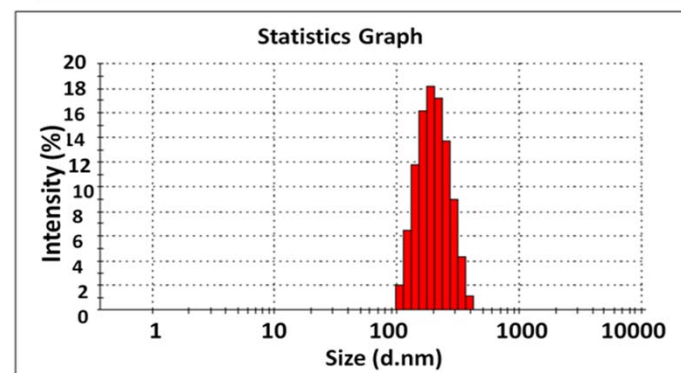
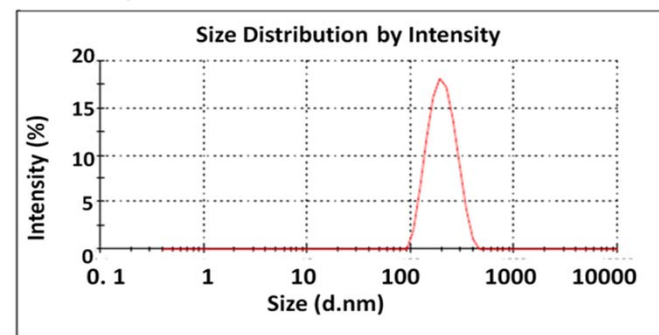


FIG. 1

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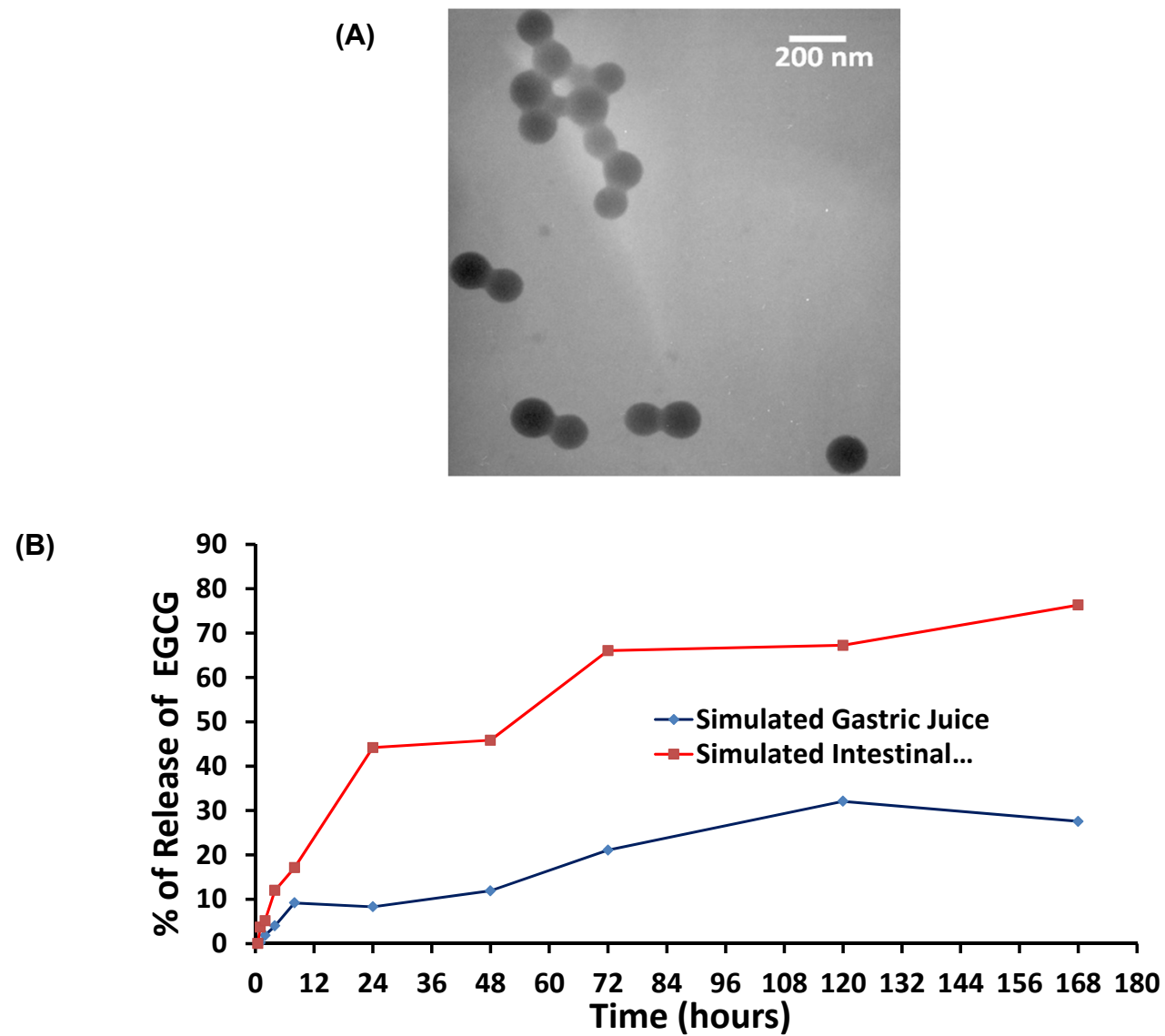


FIG. 2

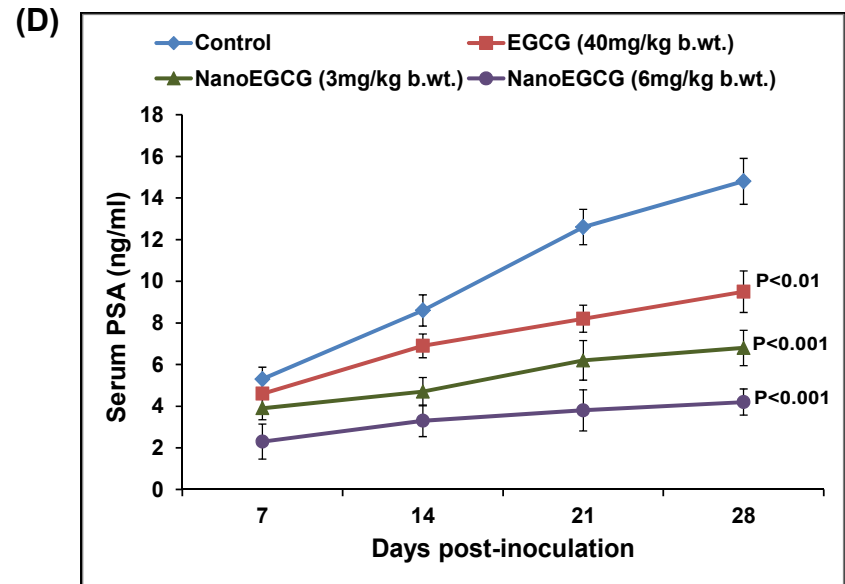
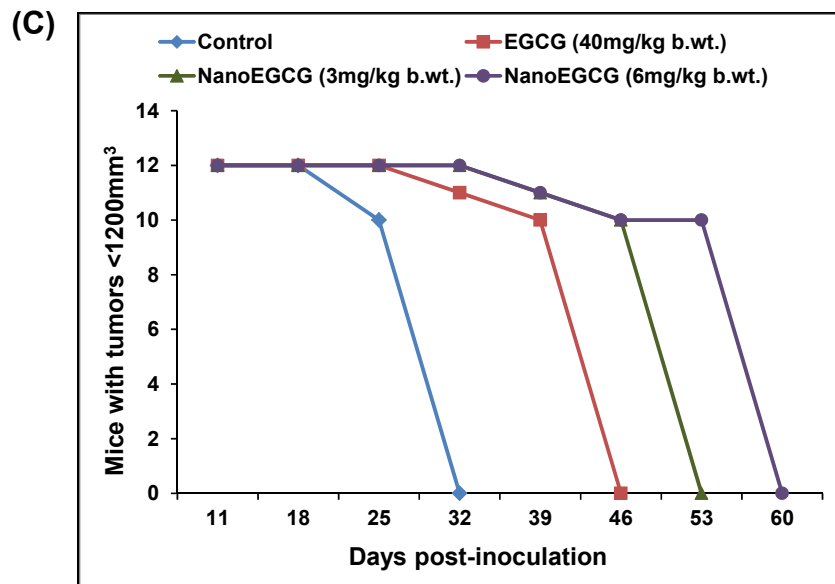
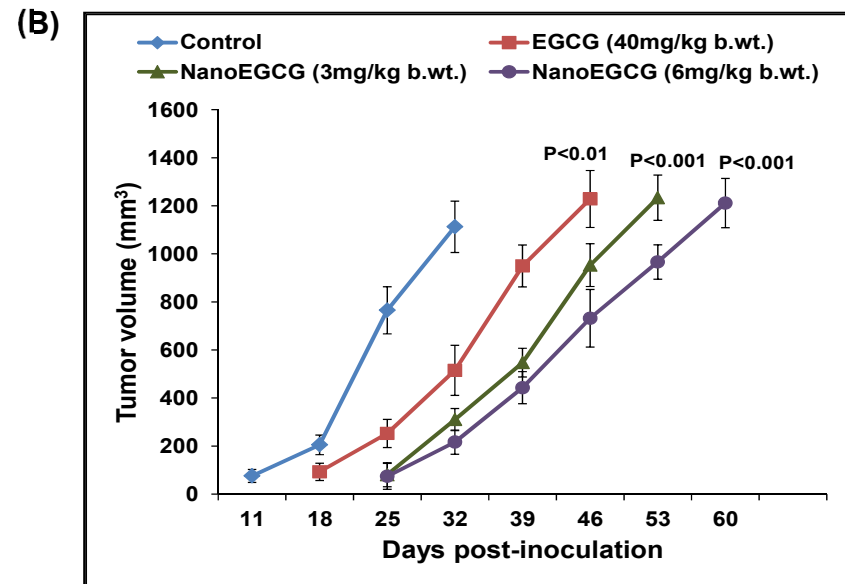
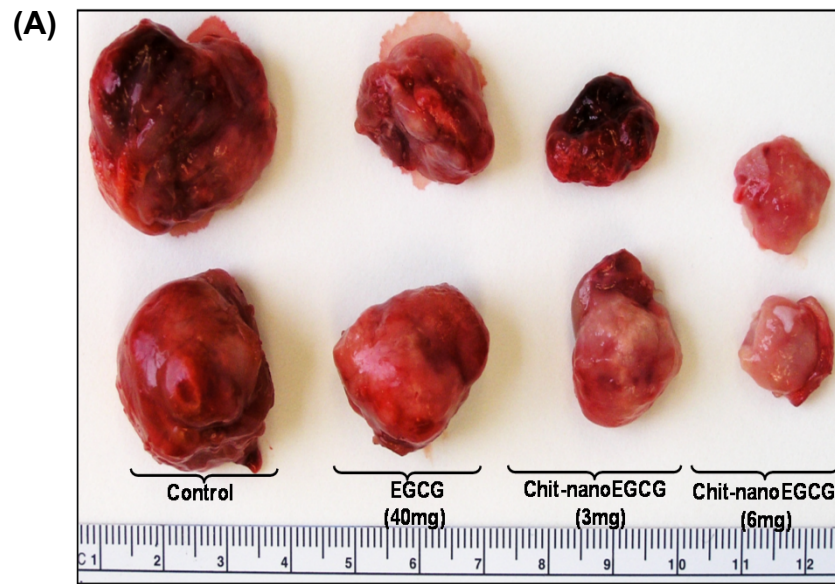


FIG. 3

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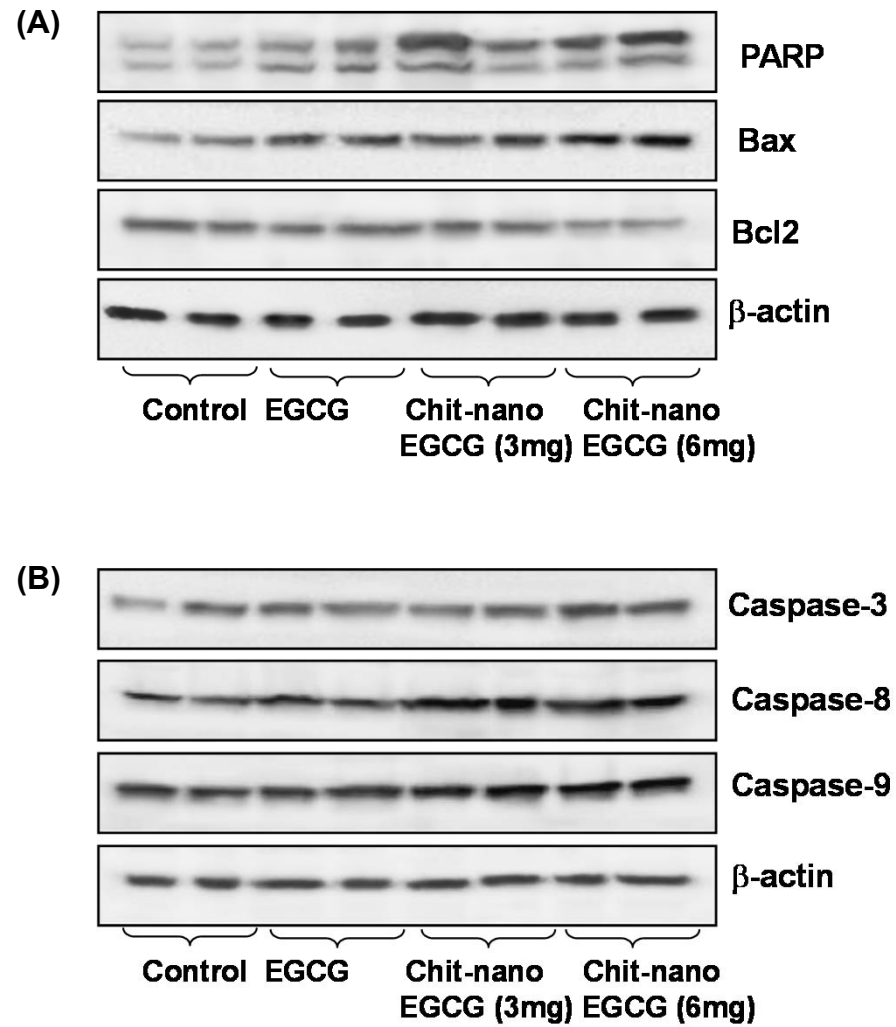


FIG. 4

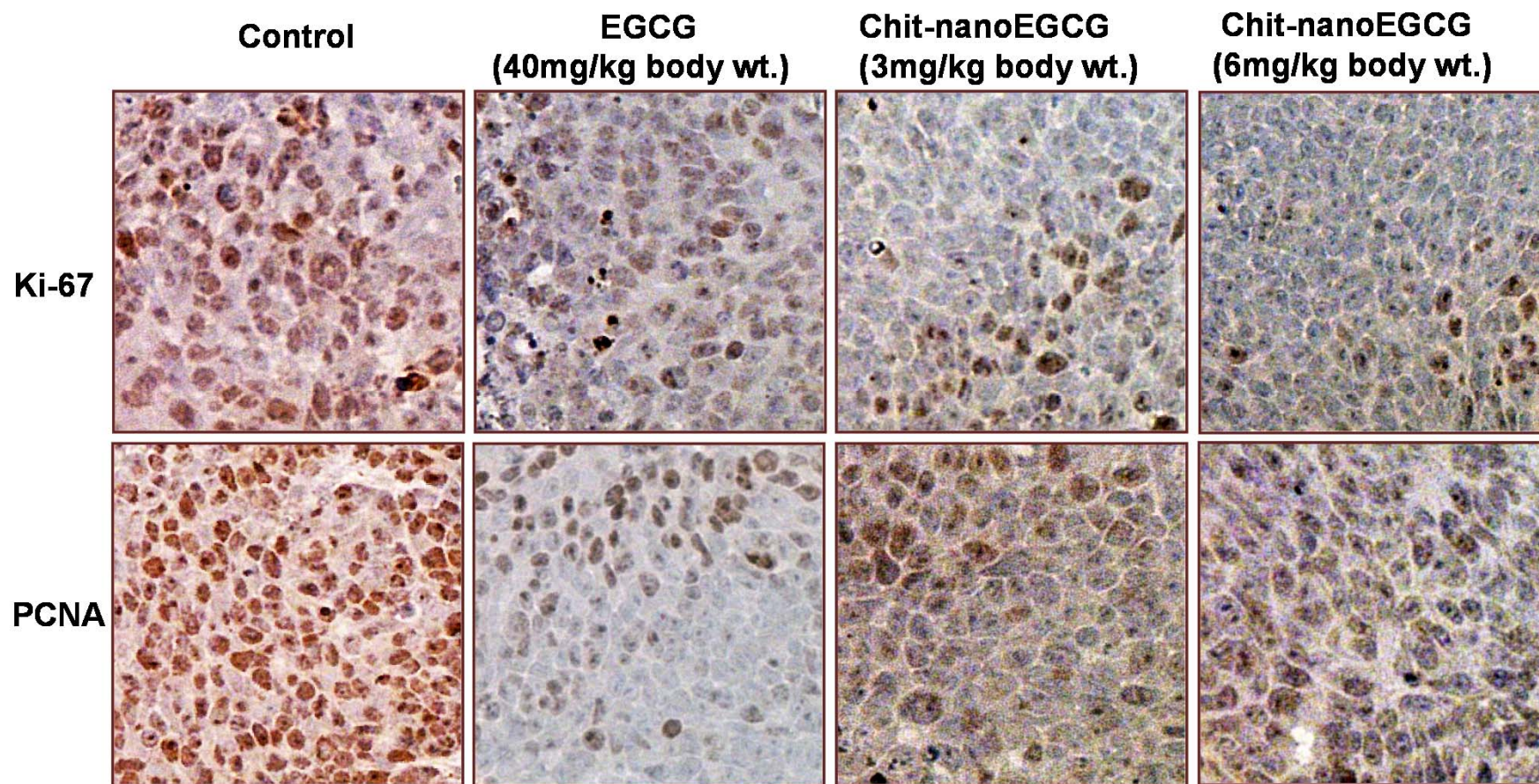


FIG. 5

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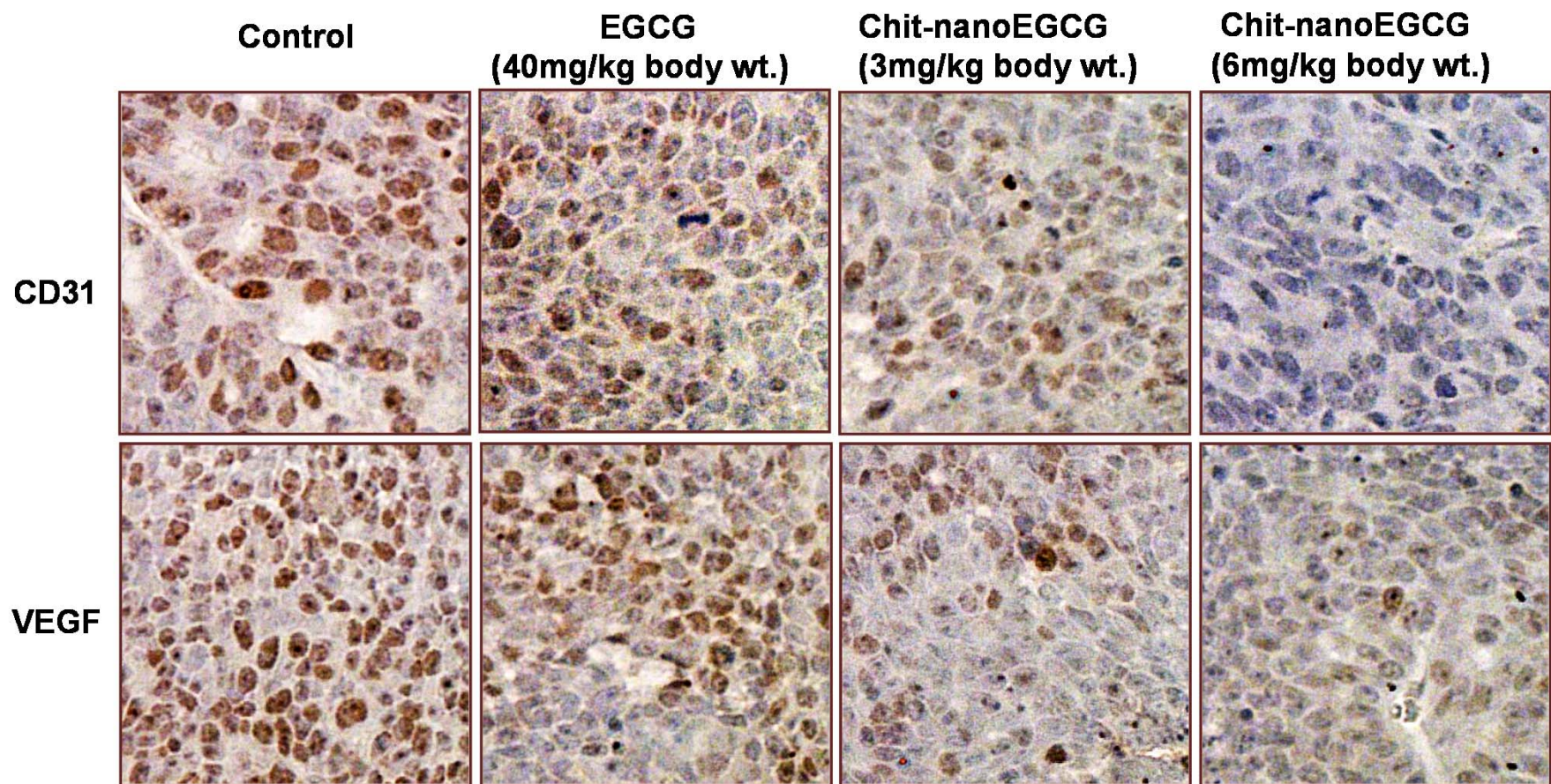


FIG. 6

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